Universidade Federal de Minas Gerais
Instituto de Ciências Agrárias
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ATIVIDADE ANTIMICROBIANA E MODULATÓRIA DO ÓLEO ESSENCIAL MICROENCAPSULADO DE *SYZYGIUM AROMATICUM* EM *STAPHYLOCOCCUS AUREUS* MULTIRRESISTENTES

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Área de Concentração: Manejo e criação de animais

Orientador: Anna Christina de Almeida

Coorientadores: Caroline Magalhães Caires, Charles Martins Aguilar, Ivan Pires de Oliveira, Cintya Neves de Souza

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ATA DE DEFESA DE DISSERTAÇÃO

Aos 08 dias do mês de agosto de 2025 às 14:00 horas, sob a Presidência da Professora Anna Christina de Almeida, D. Sc. (Orientadora – UFMG/ICA) e com a participação da Técnica-Administrativa Cintya Neves de Souza , D. Sc. (Coorientadora
- UFMG/ICA), da Professora Eliane Macedo Sobrinho Santos, D. Sc. (IFNMG – Campus Araçuaí) e da Pós-Doutoranda Geziella Aurea Aparecida Damasceno Souza,
D. Sc. (Unimontes), reuniu-se, por videoconferência, a Banca de defesa de dissertação de **Ellem Cristina Gomes Damascena**, aluna do Curso de Mestrado em Produção Animal. O resultado da defesa de dissertação intitulada **"Atividade antimicrobiana e modulatória do óleo essencial microencapsulado de *Syzygium aromaticum* em *Staphylococcus aureus* multirresistentes"** sendo a aluna considerada **APROVADA**. E, para constar, eu, Professora Anna Christina de Almeida, Presidente da Banca, lavrei a presente Ata que depois de lida e aprovada, será assinada por mim e pelos demais membros da Banca examinadora.

OBS.: A aluna somente receberá o título após cumprir as exigências do ARTIGO 53 do regulamento e da resolução 05/2016 do Curso de Mestrado em Produção Animal.

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RESUMO

Óleos essenciais (OEs) tem sido utilizados no controle de diversos microrganismos, como a bactéria *S. aureus*, uma das responsáveis pela mastite bovina. Apesar de eficaz, sua baixa estabilidade físico-química limita o uso prático, tornando a microencapsulação uma estratégia para proteger seus compostos voláteis, aumentando a estabilidade durante a manipulação, processamento e armazenamento. Assim, esta pesquisa tem como objetivo avaliar a atividade antimicrobiana e modulatória do óleo essencial de *Syzygium aromaticum* livre e microencapsulado (OESAM) frente a bactérias multirresistentes. Foram selecionadas 24 cepas de *Staphylococcus aureus*. Foram realizados antibiogramas, testes de sensibilidade com diferentes concentrações do OE puro e do OE microencapsulado, avaliação de sua modulação, além de buscar compreender o papel do isolado proteico de soro de leite WPI 90%, utilizado na parede das microcápsulas, na potencialização dos antimicrobianos, por meio de análises de bioinformática. O perfil de resistência para a Ampicilina + Sulbactam foi de 95,83% dos isolados, seguida por Meropenem (87,5%), Oxacilina (79,16%), Cefoxitina (37,5%) e Imipenem (4,16%). A análise por cromatografia gasosa com espectrometria de massas (CG-EM) identificou o eugenol como o principal componente do óleo essencial de cravo. Com relação aos testes de sensibilidade, a concentração inibitória mínima (CIM) e a concentração bactericida mínima (CBM) para o antibiótico oxacilina apresentou valores de CIM variando de 40 a 60 µl/ml e valores de CBM de 40 a 110 µl/ml. O meropenem apresentou valores de CIM entre 5 e 40 µl/ml e valores de CBM entre 10 e 60 µl/ml. O óleo essencial apresentou CIM de 10 µl/ml para 100% das cepas testadas e CBM de 20 µl/ml e 40 µl/ml. E a atividade moduladora foi avaliada pelo método do tabuleiro de xadrez, revelando uma redução de 16 vezes nos valores de CIM dos antibióticos e uma redução de 4 a 8 vezes nos valores de CIM do óleo essencial. Foi avaliado ainda o efeito do (OESAM) utilizando a técnica de coacervação complexa no processo de microencapsulação. A atividade inibitória do (OESAM), associado ou não a antibióticos beta-lactâmicos, foi testada contra 10 isolados de cepas multirresistentes de *S. aureus*. Foi observada diferença significativa nas concentrações inibitórias mínimas dos antibióticos oxacilina e meropenem isoladamente e combinados com OESAM ($p<0,05$). Houve redução média de 12 e 51µg.ml-1 na concentração inibitória mínima do OESAM quando combinado com os antibióticos oxacilina e meropenem, respectivamente. Houve redução média nas doses antimicrobianas de até 2,7 vezes quando combinado com OESAM. Embora em algumas cepas de *S. aureus* tenha sido observado efeito indiferente entre OESAM e os antibióticos, os efeitos de sinergismo e aditividade se destacaram. A análise bioinformática revelou três clusters na rede de interação proteica. Este estudo demonstrou o potencial do uso de OESA e OESAM combinado com antibióticos beta-lactâmicos no

combate a cepas multirresistentes de *S. aureus*, mitigando os mecanismos de resistência dessas bactérias aos antimicrobianos convencionais.

Palavras- chave: Cravo da Índia; microencapsulação; modulação; resistência antimicrobiana, bactérias gram- positivas.

ABSTRACT

Essential oils (EOs) are secondary metabolites extracted from different parts of plants, but their low physicochemical stability limits their practical use. To overcome this problem, microencapsulation has been used as a strategy to protect their volatile compounds, increasing stability during handling, processing and storage. The aim of this study was to evaluate the antimicrobial and modulatory activity of free and microencapsulated *Syzygium aromaticum* essential oil (OESAM) against multidrug-resistant bacteria. Twenty-four strains of *Staphylococcus aureus* from different herds in the north of Minas Gerais were selected by MALDI-TOF MS mass spectrometry. Antibiograms, sensitivity tests with different concentrations of pure EO and microencapsulated EO, evaluation of their modulation, as well as seeking to understand the role of whey protein isolate WPI 90%, used in the wall of microcapsules, in potentiating antimicrobials, were carried out using bioinformatics analysis. The resistance profile for Ampicillin + Sulbactam was 95.83% of the isolates, followed by Meropenem (87.5%), Oxacillin (79.16%), Cefoxitin (37.5%) and Imipenem (4.16%) . Analysis by gas chromatography with mass spectrometry (GC-MS) identified eugenol as the main component of clove essential oil. With regard to sensitivity tests, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for the antibiotic oxacillin showed MIC values ranging from 40 to 60 µl/ml and MBC values from 40 to 110 µl/ml. Meropenem showed MIC values between 5 and 40 µl/ml and CBM values between 10 and 60 µl/ml. The pure oil showed a MIC of 10 µl/ml for 100% of the strains tested and a CBM of 20 µl/ml and 40 µl/ml. And the modulating activity was evaluated using the checkerboard method, revealing a 16-fold reduction in the MIC values of the antibiotics and a 4 to 8-fold reduction in the MIC values of the essential oil. The effect of (OESAM) was also evaluated using the complex coacervation technique in the microencapsulation process. The inhibitory activity of (OESAM), associated or not with beta-lactam antibiotics, was tested against 10 isolates of multidrug-resistant strains of *S. aureus*. A significant difference was observed in the minimum inhibitory concentrations of the antibiotics oxacillin and meropenem alone and combined with OESAM ($p<0.05$). There was an average reduction of 12 and 51µg.ml-1 in the minimum inhibitory concentration of OESAM when combined with the antibiotics oxacillin and meropenem, respectively. There was an average reduction in antimicrobial doses of up to 2.7 times when combined with OESAM. Although in some *S. aureus* strains an indifferent effect was observed between OESAM and the antibiotics, the effects of synergism and additivity stood out. Bioinformatic analysis revealed three clusters in the protein interaction network. This study demonstrated the potential of using OESA and OESAM in combination with beta-lactam antibiotics to

combat multi-resistant strains *of S. aureus*, mitigating the resistance mechanisms of these bacteria to conventional antimicrobials.

Keywords: Clove; microencapsulation; modulation; antimicrobial resistance, gram-positive bacteria

ABREVIATURAS E SIGLAS

µg: micrograma

µL: microlitro

ATCC: American Type Culture Collection

CIM :Contagem inibitória mínima

CBM :Contagem bactericida mínima

CLSI: Clinical and Laboratory Standards Institute

CMT: (*California Mastitis Test*)

ICA: Instituto de ciências agrárias

MAPA: Ministério da Agricultura, Pecuária e Abastecimento

OE: Óleo essencial

OESA : Óleo essencial de *Syzygium aromaticum*

OESAM :Óleo essencial de *Syzygium aromaticum*

UFMG :Universidade Federal de Minas Gerais

SUMÁRIO

1. INTRODUÇÃO GERAL

Syzygium aromaticum popularmente conhecido como cravo-da-Índia é tradicionalmente empregado no tratamento de várias doenças e infecções, e acredita-se que possa substituir alguns antibióticos no combate a essas condições futuramente. O efeito sinérgico da combinação de antibióticos com extratos vegetais contra bactérias resistentes abre novas possibilidades para o tratamento de enfermidades infecciosas. Esse efeito potencializa a ação do antibiótico específico, impedindo que ele perca sua eficácia (AJOBIEWE et al., 2022).

Os antibióticos beta-lactâmicos são frequentemente utilizados no tratamento da mastite bovina, uma das enfermidades mais preocupante na bovinocultura de leite (SRIVASTAVA; KUMARESAN, 2015). Trata-se de uma inflamação nas glândulas mamárias, frequentemente provocada por infecções causadas por diversos tipos de microrganismos, como o *Staphylococcus aureus*. Essa doença está entre os maiores desafios enfrentados pela bovinocultura leiteira devido à elevada ocorrência de casos clínicos, à grande incidência de infecções subclínicas e aos significativos impactos econômicos que gera (CADES et al., 2017).

Neste cenário, a resposta insatisfatória ao tratamento com antibióticos e o aumento da resistência bacteriana, especialmente em *S.aureus*, têm sido o interesse de muitos estudos que visam identificar os fatores responsáveis pela falha do tratamento, a fim de tornar os tratamentos futuros mais eficazes (FANIN *et al*., 2020). Dentre as formas mais preocupantes está a resistência bacteriana a meticilina, cefoxitina e/ou oxacilina (LOCATELLI *et al*., 2017).

Considerando a resistência microbiana a diversos antibióticos comumente empregados, pesquisas usando óleos essenciais (OE) despertam o interesse cada vez mais dos pesquisadores que buscam evidências de sua eficácia e sua ação antimicrobiana. São considerados seguros quando utilizados em concentrações adequadas e reduzem os parâmetros de isolamento e purificação dos compostos, além de possuírem alta atividade antimicrobiana, sendo reconhecidos como seguro pela Food and Drug Administration (FDA, 2017).

Apesar de suas vantagens, o OE têm uma grande limitação com relação a instabilidade na presença de luz, calor, oxigênio e umidade. Portanto, métodos de microencapsulação são amplamente utilizados para evitar que o OE seja degradado por estes fatores externos, aumentando assim sua estabilidade (JUGREET *et al*., 2020). O processo de microencapsulação geralmente consiste em revestir partículas de um material com uma película fina de outro composto, como um polímero, formando estruturas denominadas de microcápsulas (GÜNDEL *et al*., 2018).

Syzygium aromaticum, popularmente conhecido como cravo-da-Índia, é tradicionalmente empregado no tratamento de várias doenças e infecções. Tem sido empregado desde a antiguidade por suas propriedades antiespasmódicas, antibacterianas e antifúngicas, enquanto os botões florais eram utilizados utilizados no manejo de distúrbios gastrointestinais. Seu principal componente, o eugenol, desperta crescente interesse científico devido ás atividades anti-inflamatórias, quimiopreventivas e antimicrobianas já evidenciadas (KAMATOU *et al*.,2012).

Modificadores de atividade antibiótica é um termo aplicado a substâncias que modulam ou revertem a resistência antimicrobiana a antibióticos específicos, ou seja, quando diversos produtos naturais como extratos e fitoconstituintes modificam a sensibilidade bacteriana a antibióticos, por exemplo, inibição de bombas de efluxo (MORAIS *et al*., 2021;COUTINHO *et al*.,2015).

O efeito sinérgico da combinação de antibióticos com extratos vegetais contra bactérias resistentes abre novas possibilidades para o tratamento de enfermidades infecciosas. Esse efeito possibilita a utilização do antibiótico específico quando ele não for mais eficaz de forma isolada durante a terapia (AJOBIEWE *et al*.,2022).

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar a atividade antimicrobiana e modulatória do óleo essencial microencapsulado de *Syzygium aromaticum* em *Staphylococus aureus* multirresistentes.

2.2 Ojetivos específicos

- Identificar espécies de *Staphlylococcus aureus* em isolados de leite mastítico e selecionar multirresistentes a antibióticos da classe dos beta-lactâmicos.
- Identificar e validar a composição da amostra de óleo adquirido comercialmente pela técnica de cromatografia gasosa aclopada á Espectrometria de massas (CG-MS).
- Realizar a microencapsulação do óleo essencial de *Syzygium aromaticum* e avaliar a sua eficácia.
- Verificar a atividade antimicrobiana *in vitro* do óleo essencial de *Syzygium aromaticum* (OESA) e do óleo essencial *Syzygium aromaticum* microencapsulado (OESAM).
- Avaliar a potencialidade do óleo essencial de *Syzygium aromaticum* (OESA) e do óleo essencial *Syzygium aromaticum* microencapsulado (OESAM) na modulação da resistência antimicrobiana de *Staphylococus aureus* multirresistentes.
- Investigar mecanismos moleculares envolvidos na atuação do OESAM por meio de análise de bioinformática.

3. REVISÃO DE LITERATURA

3.1 Mastite bovina

A bovinocultura de leite vem crescendo no Brasil progressivamente. Os primeiros dados da produção leiteira foram registrados pela Organização das Nações Unidas para a Alimentação e a Agricultura (FAO) em 1961, a qual contabilizou 5,2 milhões de litros (VIELA *et al*., 2017). Atualmente, esse número vem crescendo de maneira gradual, e no ano de 2024, a aquisição de leite cru feita pelos estabelecimentos que atuam sob algum tipo de inspeção sanitária (Federal, Estadual ou Municipal) foi de 25,38 bilhões de litros (IBGE, 2024).

Uma das enfermidades mais preocupante na bovinocultura de leite, a mastite bovina, provoca queda de produção, causando prejuízos econômicos significativos. É uma afecção resultante de um processo infeccioso e inflamatório da glândula mamária (MORITZ;FENIMAN,2016), possuindo

classificação em clínica e subclínica. A forma clínica causa alterações no leite e no úbere, enquanto a subclínica é caracterizada por aumento na contagem de células somáticas (CCS) no leite, sem manifestações visíveis no animal (BAKRY *et al*.,2016), no qual só é possível o diagnóstico, através de métodos indiretos como o CMT (*California Mastitis Test*) (ADKINS; MIDDLETON, 2018).

Os fatores de riscos no animal estão relacionados a idade, imunossupressão, número de partos, estágio de lactação, entre outros. Já os fatores externos estão relacionados a higienização do úbere, o material utilizado na cama e regulagem da ordenhadeira mecânica. Devido a relevância da mastite, o conhecimento sobre a prevalência, principais patógenos e fatores de risco têm auxiliado indústrias de laticínios na elaboração de medidas preventivas e controle (SONG *et al*., 2020).

O tratamento da mastite clínica deve ser imediato, portanto a realização de antibiogramas antes da aplicação em campo é necessário para um tratamento mais assertivo e evitar resistência antimicrobiana. Em casos septicêmicos, de maior magnitude, é recomendado tratamento sistêmico (RIBEIRO *et al*., 2016).

3.2 Staphylococcus sp. em saúde animal e humana

O gênero *Staphylococcus*, pertencente à família Staphylococcaceae, abriga 69 espécies e 30 subespécies de *Staphylococcus* classificadas em: coagulase positiva e coagulase negativa (PARTE *et al*.,2020). A heterogeneidade evidente no gênero levou a uma classificação baseada em procedimentos de diagnóstico com a característica de produção de coagulase, facilitando a abordagem clínica ao diferenciar entre espécies patogênicas *S. aureus* e um grupo de estafilococos inicialmente classificado como não patógeno, os estafilococos coagulase negativa (SILVA, 2018).

Staphylococcus aureus pertence ao grupo dos cocos, isto é, possuem o formato esférico, onde estão organizadas em cachos de uvas. Caracterizam-se pela coloração amarelada de suas colônias e desempenham suas atividades metabólicas em ambiente com a presença de oxigênio ou sem a presença deste, denominado anaeróbios facultativos. São pertencentes da microbiota normal da maior parte dos animais domésticos e, nestes, podem atuar como

agentes patogênicos oportunistas, promovendo várias afecções, representando uma problemática em saúde única em virtude da interação da tríade, homem, animal e meio ambiente (SOUZA *et al*., 2017; TORTORA *et al*., 2017).

Nos diversos contextos agropecuários ao redor do mundo, os principais reservatórios de *S. aureus* são animais de produção, como suínos, bovinos, pequenos ruminantes e aves, os quais desempenham papel central na cadeia de transmissão. Uma característica particularmente preocupante de *S. aureus* é sua notável plasticidade genômica, que possibilita a colonização e infecção de múltiplos hospedeiros, além de contribuir para a ocorrência de eventos zoonóticos. A principal via de exposição humana ocorre por meio do consumo de produtos de origem animal contaminados, como leite, carne e ovos, evidenciando a necessidade de medidas rigorosas de controle sanitário ao longo de toda a cadeia produtiva. Assim, a adoção de estratégias integradas de vigilância epidemiológica, uso racional de antimicrobianos e boas práticas agroindustriais constitui um elemento essencial para mitigar os impactos causados por esse patógeno (PAL *et al*., 2020). S *aureus* é um patógeno de alta relevância médica e veterinária, cuja virulência decorre da produção de toxinas associadas à evasão imune e à gravidade das infecções. Enterotoxinas estão ligadas à intoxicação alimentar, enquanto TSST-1 e toxinas esfoliativas causam síndromes sistêmicas graves, e as toxinas hemolíticas (α, β, δ e γ) promovem destruição celular e disseminação bacteriana. A compreensão desses mecanismos é essencial para subsidiar estratégias preventivas e terapêuticas eficazes (PEREIRA *et al*., 2024).

3.3 Antibióticos da classe dos Beta-lactâmicos

Antibióticos são substâncias que são desenvolvidas através de microrganismos ou sintetizadas quimicamente que podem causar a morte de outros microrganismos. Quando impedem o crescimento bacteriano são denominados bactericidas, ou quando impedem a replicação de bactérias estão agindo como bacteriostáticos (VERMELHO *et al.*, 2019).

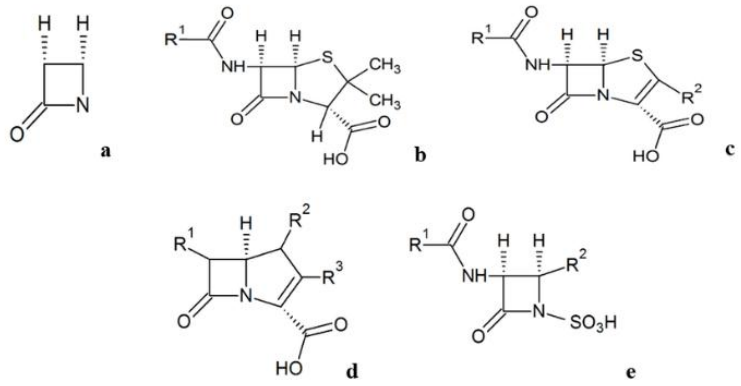
Os beta-lactâmicos constituem a maior classe de antibióticos e são amplamente utilizados na prática clínica humana e de animais. Todos os compostos deste grupo (penicilinas, cefalosporinas, carbapenêmicos,

monobactâmicos e inibidores de beta-lactamases) possuem um anel beta-lactâmico característico e diferentes cadeias laterais, o que explica as propriedades e o espectro de ação de cada antimicrobiano. Tem efeito bactericida lento e sua ação é dependente do tempo. Geralmente apresenta boa distribuição e baixa toxicidade. No entanto, nenhum beta-lactâmico chegou ao mercado em mais de 30 anos (GUERRA, 2020).

O grupo dos beta- lactâmicos é caracterizado por possuir um anel beta-lactâmico na molécula dos antimicrobianos pertencentes ás subclasses: penicilinas, cefalosporinas, carbapenêmicos, monobactâmicos e inibidores de beta-lactamase (FARIAS et al.,2022) (Figura 1). O anel beta-lactâmico é composto por três átomos de carbono e um átomo de nitrogênio, mas para ser ativado, deve estar ligado a outro anel, geralmente pela presença de um radical em sua estrutura química (ARRUDA *et al*.,2019).

A maioria dos beta-lactâmicos são ácidos fracos, solúveis em gordura facilmente absorvidos em pH baixo. Este grupo de antibióticos atravessa a membrana plasmática através de transporte ativo ou de difusão passiva (ARRUDA *et al*.,2019).

Figura 1- Estruturas moleculares dos beta-lactâmicos. (a) Anel beta-lactâmico, (b) Penicilina, (c) Cefalosporina, (d) Carbapenêmico, (e) Monobactâmico



Fonte: ARAÚJO; *et al.*, 2020.

3.3.1 Mecanismo de resistência aos beta-lactâmicos

A resistência aos antimicrobianos é um desafio que está presente nos debates e diretrizes de saúde pública no mundo. Ela ocorre quando microrganismos como bactérias e fungos passam por modificações no decorrer do tempo e os fármacos que anteriormente tinham efeito terapêutico sobre eles, se revelam ineficazes (WHO, 2021). Algumas modificações estão relacionadas à mutações aleatórias e espontâneas que ocorrem por meio do processo de replicação bacteriana, que modificam os genes podendo transformar as características morfológicas e metabólicas das células, ocasionando perda na eficiência do antimicrobiano (FARIA *et al.*, 2022).

Diversas espécies de microrganismos apresentam multirresistência a antimicrobianos, incluindo penicilina, vancomicina e β-lactâmicos, comprometendo não apenas a eficácia dos tratamentos clínicos, mas também representa um desafio à saúde pública e à produção animal, resultando em perdas econômicas e riscos à segurança alimentar (PAL *et al*., 2020).

As enzimas beta-lactamases têm a capacidade de hidrolisar o anel beta-lactâmico. Essas enzimas são codificadas nos plasmídeos, transpósons e íntegrons, que podem carrear o gene de resistência transmitindo horizontalmente para diferentes bactérias (LIMA *et al.,* 2017).

Entre os fatores que contribuem para o desenvolvimento da resistência microbiana destacam-se o descarte inadequado de frascos, seringas ou outros veiculadores de resíduos de antibióticos, afetando de maneira geral a saúde animal e humana, a venda de antimicrobianos falsificados e de baixa qualidade e o uso indiscriminado de antibióticos (MARQUES *et al*., 2023).

3.4 Staphylococcus spp. Multirresistentes

Todos os microrganismos que não têm seu crescimento e multiplicação inibido pelas concentrações dos fármacos no sangue ou tecidos, ou aqueles que não possuem resposta efetiva no decorrer do tratamento, são classificados como resistentes, e já aqueles que têm a capacidade de resistir aos efeitos de vários antibióticos são chamados de multirresistentes, seja por uma característica específica às bactérias ou adquirida ao longo de uma infecção (PONS *et al*., 2020).

Algumas bactérias multirresistentes vêm tornando prevalentes em infecções comunitárias uma vez que o desenvolvimento de novos medicamentos para combater essas bactérias é lento e incerto. Isso tem um impacto negativo na vida das pessoas, dos animais e na qualidade dos alimentos (MIQUET, 2017;CUNY *et al.*, 2017).

A resistência bacteriana afeta tanto as pessoas hospitalizadas quanto a microbiota do ambiente hospitalar, devido ao uso rotineiro de antibióticos de amplo espectro ou do uso de múltiplos antimicrobianos para prevenir infecções. De acordo com Bôtelho et al. (2022), a prevalência de S. *aureus* resistentes a oxacilina em amostras de pacientes, profissionais de saúde e superfícies hospitalares, foi observada na maioria dos artigos analisados.

No contexto animal, Talim et al. (2021) investigaram suinoculturas com o objetivo de caracterizar geneticamente e avaliar o potencial zoonótico de S. *aureus* multirresistentes isolados de amostras de mucosa nasal de suínos e trabalhadores. O estudo demonstrou ampla resistência, incluindo à penicilina G e a outros antibióticos β-lactâmicos, evidenciando o risco zoonótico associado a essas cepas.

3.5 Legislação de resíduos dos antibióticos e Medicamentos Fitoterápicos

A Organização Mundial da Saúde (OMS), em parceria com os países que compõem a Organização das Nações Unidas (ONU), incluindo o Brasil, incentiva a criação de planos nacionais com medidas para combater e controlar a resistência microbiana. Dentre essas medidas, destaca-se a implementação de programas que promovem o uso consciente de antimicrobianos na saúde humana e animal (ANVISA, 2023).

O Plano Nacional de Controle de resíduos e contaminantes PNCRC/Animal é um dispositivo de gerenciamento de risco, estabelecido pelo Ministério da Agricultura, Pecuária e Abastecimento (MAPA), com o intuito de proporcionar segurança química dos alimentos de origem animal produzidos no Brasil. O plano apresenta como base, a Normativa SDA n°42, implementada no dia 20 de dezembro de 1999. Neste programa são desenvolvidos planos anuais de amostragem para testes em ovos, leite e mel, através da Inspeção Federal onde serão encaminhadas para laboratórios da rede nacional de laboratórios agropecuários (MAPA, 2017).

Visando ainda a saúde única, foi estabelecida a Recomendação do Conselho Nacional de Saúde n°021, no dia 13 de julho de 2022. Foram incluídas ações para a monitoração de microrganismos resistentes e resíduos antimicrobianos em alimentos, com caráter permanente e de implementação progressiva, de responsabilização do Ministério da Saúde (CNS, 2022).

Com relação a legislação que envolve o uso de fitoterápico em humanos, gerenciado pela Agência Nacional de Vigilância Sanitária (ANVISA), vinculada ao Ministério da Saúde, uma série de normas e procedimentos foram editados, para regular, organizar e desenvolver o setor de fitoterápicos para saúde humana no País (BRUNO *et al*.,2016). A legislação mais recente é a resolução RDC n°26, de 13 de maio de 2014, que dispõe sobre o registro de medicamentos fitoterápicos, fornecendo ainda uma lista de espécies que não podem ser utilizadas na composição de produtos tradicionais fitoterápicos (ANVISA, 2014).

No setor veterinário, a regulamentação é de responsabilidade do MAPA, que regulamenta produtos veterinários, incluindo os de origem vegetal. No entanto, existem poucos padrões para a fitoterapia para animais. No caso de registros de fitoterápicos, deve-se seguir as normas de produtos, no qual a documentação deve estar de acordo com a portaria n°301 de 19 de abril de 1996 (BRUNO *et al*.,2016).

3.6 Cravo- da- Índia e sua ação antimicrobiana

O craveiro-da-índia, de nome científico (*Syzygium aromaticum* e/ou *Caryophyllus aromaticus L*.), pertence à família *Myrtaceae* com origem na Indonésia. No Brasil, a planta se adaptou muito bem no Sul da Bahia, tornando-o o estado que mais produz esta especiaria. A copa do craveiro verde, normalmente tem formato piramidal e, de seus botões florais secos podem ser extraídos o óleo, como observado na Figura 2. (HORVAT;MIYASAKA,2019; NASCIMENTO,2012).

Figura 2. Botões florais antes e depois da secagem

Fonte: Escola de botânica,2021.

Além de ter ação antimicrobiana (Quadro 1) e anticancerígena, (SHEHABELDINE *et al*.,2023) ele ainda pode atuar como conservante químico de bebidas lácteas fermentadas, por não apresentar toxicidade e reduzir níveis glicêmicos (SOUZA *et al*,2021).

Quadro 1. Ação antimicrobiana e atividades relacionadas ao cravo-da-Índia

Locais de ação	**Resultados**	**Referências**
Filme de quitosana contendo nanotubos de halloysita	Excelentes aplicações na indústria de embalagens de alimentos devido às suas altas propriedades de bloqueio de UV e também às atividades antibacterianas e antioxidantes eficazes	SAADAT *et al*.,2022

Cepas de *S. aureus e P. aeruginosa*	Ruptura da membrana bacteriana, levando à liberação significativa de proteínas das células Bacterianas	AL-MIJJALI *et al*.,2023
Biofilme	Diminuiu significativamente o desenvolvimento de biofilme;	SHEHABELDINE *et al*.,2023
Infecções em ratos causadas por resistentes à meticilina *S.aureus*	O óleo de cravo-da-índia sozinho ou em combinação com antibiótico betalactâmico imipeném curou feridas mais rapidamente e reduziu a carga microbiana nas feridas	ALANAZI *et al*.,2022
S.aureus	Os extratos vegetais apresentaram atividade antimicrobiana no isolado utilizado. concentração inibitória mínima (CIM) foi de 0,24mg/ml, e a concentração bactericida mínima (MBC) foi de 3,91mg/ml.	AJOBIEWE *et al*.,2022

Fonte: Elaborada pelo autor, 2023.

Diante disso, várias pesquisas evidenciam um potencial de atividade bactericida ou bacteriostáticas para controle de *Staphylococcus* spp utilizando métodos alternativos quando comparado á medicações comerciais. Devido ao aumento no número de cepas resistentes e multirresistentes aos antibióticos

nos últimos anos, se fez necessário a busca por novos tratamentos revolucionários, e sabe-se que num futuro próximo somente será efetivo com a utilização dessas tecnologias (FANIN *et al*., 2020).

3.7 Microencapsulação

Em virtude da baixa estabilidade físico-química do óleo essencial, a qual dificulta seu uso, o encapsulamento tem sido uma estratégia utilizada para proteger os compostos voláteis do OE, aumentando assim a estabilidade do mesmo durante a manipulação, processamento e armazenamento (BAKRY *et al.,*2016;VEIGA *et al*.,2015).

Diversas técnicas são utilizadas para a microencapsulação, desde as mais acessíveis como a técnica de emulsificação-evaporação do solvente e coacervação, a mais avançada como o método de polimerização e spray drying (RIBEIRO;VELOSO, 2021).

Considerando as limitações de alguns métodos e as propriedades físico-químicas dos óleos essenciais, a coacervação surge como técnica eficiente de microencapsulação. Ela pode ocorrer em fase aquosa, adequada para materiais hidrofóbicos, ou em fase orgânica, indicada para materiais hidrossolúveis. A coacervação aquosa pode ser simples, induzida por eletrólitos, não solventes ou variações de temperatura, ou complexa, baseada na interação de polieletrólitos com cargas opostas, sendo a formação da fase coacervada dependente de pH e força iônica (MALEŠEVIĆ *et al*., 2016).

A coacervação é um dos métodos de encapsulamento mais antigos e mais utilizados, modifica o pH, força iônica, temperatura e solubilidade sob condições controladas (TIMILSENA *et al*.,2020). Esse método é dividido em quatro etapas: A suspensão, na fase líquida, das partículas do material que irão compor o núcleo. A fase da deposição do polímero líquido em torno das partículas suspensas, a gelificação e a solidificação da parede da microcápsula (NAPIÓRKOWSKA;KUREK, 2022).

Trabalhos com o objetivo de avaliar ação antibacteriana do OESA e OESAM vêm mostrando resultados positivos, demonstrando um controle alternativo de *S. aureus,* segundo Silvia (2019) e Borborema (2022).

3.8 Plantas fitoterápicas *versus* antibióticos na modulação da resistência bacteriana

O uso de antimicrobianos derivados de plantas medicinais oferece vantagens significativas em relação aos antimicrobianos sintéticos, incluindo menor risco no desenvolvimento de resistência microbiana, maior disponibilidade e menor custo. A variedade de compostos bioativos encontrados nas plantas (Quadro 2) permite a identificação de novas substâncias com diferentes mecanismos de ação uteis no combate a microrganismos multirresistentes (BORGES,AMORIM,2020).

Quadro 2- Descrição com nome científico, nome popular, microorganismos envolvidos, composto ativo e mecanismo de ação de diferentes plantas fitoterápicas**.**

Espécie vegetal	**Nome popular**	**Microorganismos Estudados**	**Principais Compostos bioativos**	**Mecanismo de ação**	**Referências**
Punica granatum,L	Romã	*Staphylococcus aureus*	Flavonóides	Ação antioxidantepara sequestro de radicais livres.	MARTINS;CASALI,2019. OLIVEIRA *et al.*,2018.
Cymbopogoncitratus	Erva-cidreira	*Staphylococcus aureus* e*E. Coli*	Citral e Mirceno	Aumenta a permeabilidade da membrana celular, promovendo a lise da mesma, levando a morte bacteriana	SILVA *et al.*,2016. RODRIGUES *et al.*,2020. SILVA *et al.*,2014

Alliumsativum	Alho	*Staphylococcus aureus e E.coli*	Alicina ajoeno	Retarda ou inibe a multiplicação da bactéria	SILVA *et al.*,2015.
Lippiaoriganoides	Alecrim pimenta	*Staphylococcus spp.* e*Streptococcussp p*	Timol	Retarda ou inibe a multiplicação da bactéria	SOUZA *et* al.,2017.
Mimosa tenuiflora	Jurema preta	*Staphylococcus aureus*	Alcalóides, taninos e flavonoides	Inibe a síntese da parede celular bacteriana, síntese de proteínas essenciais para o crescimento dos microrganismos	SANTOS *et al.*,2022. OLIVEIRA,2023
Syzygium aromaticum.	Cravo – da-Índia	*Staphylococcus aureus*	Eugenol	Inibe a produção e amilase e proteases pela célula, promovendo sua lise e degradação	XU *et al.*,2016.

Fonte: Elaborada pelos autores, 2023.

Em busca de alternativas eficazes contra o *Staphylococcus* spp, os pesquisadores estão enfrentando grandes desafios para encontrar substâncias eficazes e com custo-benefício. O controle antimicrobiano através dos óleos essenciais, são métodos investigados por diversos autores para controlar alternativamente a ocorrência dessas bactérias. É importante ressaltar a importância do conhecimento sobre a toxicidade das plantas, ou seja, o seu risco de intoxicação em humanos e animais antes de iniciar pesquisas (FREITAS *et al*.,2021). No que se refere, óleos essenciais são substâncias

vegetais secundárias obtidas de diferentes partes das plantas. Possui composição química complexa, o que garante a planta vantagem de se adaptar ao ambiente em que está inserida (MIRANDA *et al.*, 2016; VIEIRA *et al.*, 2018).

A verificação dos elementos presentes nos óleos essenciais é feita através da técnica de cromatografia gasosa, um método que permite reconhecer os óleos, analisar os seus constituintes e determinar a sua pureza. Cada óleo essencial pode ter até 300 ou mais componentes, devido a sua grande amplitude terapêutica, tem atuação em vários sistemas no corpo, assim como na psique (WOLFFENBUTTEL, 2016).

São componentes de óleos essenciais de modo geral: limoneno, pineno, mentol, terpinen-4-ol, linalol, cinamaldeído, cetonas, verbenona, piperitona, acetato linalílico, mentofurano, timol, carvacrol, eugenol, miristicina, 1,8-cineol (eucaliptol) e o bergapteno (WOLFFENBUTTEL, 2016).

Estudos vêm sendo realizados acerca da ação antimicrobiana de OE. Na pesquisa realizada por Beraldo *et al*. (2013), observou-se que o cravo - da - Ìndia mostrou-se mais eficiente que o hipoclorito de sódio, na inibição das bactérias, utilizando-se o método da microdiluição, mostrando possível aplicação de óleos essenciais como princípios ativos de sanitizantes. Outra pesquisa realizada buscando avaliar a atividade antimicrobiana in vitro do cravo- da -Índia, os autores concluíram que houve uma resposta positiva frente as estirpes analisadas utilizando diferentes concentrações (CASTRO ;FERREIRA,2017), corroborado na pesquisa de (BAI *et al*.,2023), onde o óleo de cravo-da-Índia apresentou atividade antimicrobiana significativa de moderada a forte, quanto testado contra *Staphylococcus aureus* .

A modulação de medicamentos utilizando o OE é caracterizada pela capacidade de modificarem a ação de antimicrobianos, proporcionando assim, a inversão da resistência antimicrobiana pela destruição de plasmídeos ou pela inibição de bombas de efluxo, por exemplo. As bombas de efluxo são proteínas presentes na membrana das bactérias responsáveis por expulsar ativamente substâncias nocivas, como antibióticos, do meio intracelular, tanto em bactérias Gram-positivas quanto Gram-negativas. Elas podem atuar como bombas de resistência específica (RE) ou multirresistência (RM), influenciando a ação de uma ou mais categorias de antimicrobianos e compostos estruturalmente relacionados (MORAIS *et al*., 2021).

A associação entre produtos naturais e fármacos sintéticos possui uma grande importância, pois contribui para a redução da concentração mínima inibitória (CMI) dos antimicrobianos pela diminuição dos efeitos indesejáveis e pela ação sinérgica originada por este processo (NASCIMENTO *et al.*, 2018; FERREIRA *et al*., 2019).

No trabalho Cirino (2014) testou-se vários óleos essenciais de plantas e seus constituintes majoritários, onde perceberam que produtos naturais testados de maneira geral apresentaram atividade antibacteriana significativa, indicando que podem atuar como atividade moduladora, reduzindo a contagem inibitória mínima dos antibióticos testados de até dezesseis vezes, sob ação nas cepas de *Staphylococcus aureus*. Todas as cepas avaliadas apresentaram sensibilidade à ação composta pelo OE e dos antibióticos testados. Os resultados mostram que o uso do OE em combinação com antibióticos no combate a bactérias patogênicas é promissor.

Outro estudo avaliando a modulação do composto α-pineno presente em várias plantas fitoterápicas associado a um dispositivo de diodo emissor de luz contra bactérias multirresistentes, também obteve atividade satisfatória contra *Staphylococcus aureus*, quando combinado com antibióticos convencionais (NICOLAU *et al.*,2022).

3.9 Ánalise de Bioinformática

O estudo in sílico corresponde a uma metodologia que emprega ferramentas computacionais para simular processos biológicos, permitindo a realização de experimentos em ambiente virtual. Essa abordagem está diretamente associada à bioinformática, área dedicada ao desenvolvimento e aplicação de métodos computacionais voltados à aquisição, armazenamento, organização, análise e visualização de dados biológicos, médicos e de saúde, visando ampliar sua interpretação e aplicação científica (TAMASCO,2021; FASSLER;COOPER, 2011).

STRING é um banco de dados que reúne interações proteína–proteína conhecidas e previstas, abrangendo tanto associações diretas (físicas) quanto indiretas (funcionais). É uma ferramenta valiosa para explorar a função e as interações de proteínas em um contexto celular e pode ajudar a identificar

proteínas-chave e vias de sinalização envolvidas em eventos biológicos específicos (SHANNON *et al*., 2003).

Essas interações derivam de predições computacionais, transferência de conhecimento entre organismos e integração de informações provenientes de bases de dados primárias. Especificamente, o STRING disponibiliza dois conjuntos principais de interações, incluindo um grupo de nove espécies consideradas de alta relevância e confiabilidade (SZKLARCZYK *et al*., 2019).

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5. ARTIGO CIENTÍFICO 1- MODULATORY ACTION OF CLOVE ESSENTIAL OIL COMBINED WITH BETA-LACTAM ANTIMICROBIALS IN INHIBITING MULTIRESISTANT STRAINS OF STAPHYLOCOCCUS AUREUS

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ABSTRACT

This study aims to evaluate the modulatory activity of *Syzygium aromaticum* (clove) essential oil in combination with β-lactam antibiotics against multidrug-resistant *Staphylococcus aureus*. Susceptibility tests were conducted on isolates identified as *S. aureus* using by MALDI-TOF MS mass spectrometry. Susceptibility test were performed to beta-lactam antibiotics. The Ampicillin + Sulbactam was effective against 95.83% of the isolates, followed by Meropenem (87.5%), Oxacillin (79.16%), Cefoxitin (37.5%), and Imipenem (4.16%). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined for Oxacillin and Meropenem. Oxacillin showed MIC values ranging from 40 to 60 µl/ml and MBC values from 40 to 110 µl/ml. Meropenem had MIC values between 5 and 40 µl/ml and MBC values between 10 and 60 µl/ml. Gas chromatography–mass spectrometry (GC-MS) analysis identified eugenol as the major component of the clove essential oil. The oil showed MIC of 10 µl/ml and MBCs of 20 µl/ml and 40 µl/ml for 100% of the tested strains. Finally, modulatory activity was assessed using the checkerboard method, revealing a 16-fold reduction In the MIC values of the antibiotics and a 4-8 fold reduction in the MIC values of the essencial oil. These findings reinforce the potential of natural compounds in combating bacterial resistance. Further studies are needed to clarify their mechanisms of action and assess their safety in clinical application.

Keywords: Antibiotics, *Syzygium aromaticum*, Antimicrobial resistance, Modulation.

INTRODUCTION

According to the World Health Organization (WHO, 2024), antimicrobial resistance is considered one of the greatest global threats facing humanity today. In European region, antimicrobial resistance directly causes 133000 deaths annually and is indirectly associated with another 541000 deaths. Although resistance arises naturally over time - typically through genetic mutations - the misuse and overuse of antimicrobials in humans, animals, and agriculture have significantly accelerated this process (WHO, 2024; Morehead & Scarbrough, 2018).

In the context of animal health, the expansion of livestock herds and intensification of management practices have led to the emergence of opportunistic pathogens that frequently cause clinical conditions such as bovine mastitis. This condition is primarily caused by common udder-resident pathogens, including staphylococci, streptococci, and coliform bacteria (Massote et al., 2019). *Staphylococcus aureus* is frequently isolated from mastitic milk and is known to cause persistent infections with varying clinical severity. A public health concern associated with this pathogen is its ability to develop antimicrobial resistance and transfer resistance genes to humans via milk (Silva et al., 2018).

β-lactam antibiotics are characterized by a β-lactam ring in their molecular structure, which grants bactericidal activity to subclasses such as penicillins, cephalosporins, carbapenems, monobactams, and β-lactamase inhibitors (Farias et al., 2022). However, the emergence of β-lactamase-producing *S. aureus* has rendered these antibiotics less effective - especially when they are misused (Al-Hussaniy & Kadhim, 2022). These drugs work by penetrating bacterial cells and disrupting the synthesis of peptidoglycan in the cell wall (Durand et al., 2019).

Resistance to β-lactams such as methicillin, cefoxitin, and/or oxacillin is particularly concerning. Methicillin, in particular, has been the drug of choice in food-producing animals for strains that produce penicillinases (Locatelli et al., 2017; Hachiya, 2017; Weterings, 2017). On the other hand, antibiotics like Imipenem and Meropenem are not commonly used in food animals; they are

considered “*reserve*” antibiotics and, even in human medicine, should be used only as a last resort when no other options are effective (WHO, 2017; WHO, 2019).

Given this context, antibiotic resistance represents a global health challenge that demands innovative therapeutic approaches. Natural substances have gained attention for their antimicrobial and modulatory properties, with potential to inhibit resistance mechanisms and enhance the effectiveness of antibiotics (Nery et al., 2023).

Essential oils have recently attracted growing interest among users, researchers, and healthcare professionals. Clove (*Syzygium aromaticum*), a plant native to Indonesia and part of the Myrtaceae family, encompasses around 3,000 species of tropical and subtropical trees and shrubs with notable pharmacological relevance (Horvat, 2019). Its chemical composition includes eugenol, eugenol acetate, caryophyllene, oleanolic triterpene, plant waxes, ketones, resins, tannins, and sterols. Among these, β-caryophyllene is known for its anti-inflammatory, antitumor, bactericidal, insecticidal, and antiallergic properties (Affonso et al., 2012; Gomes et al., 2018).

In pharmacology, modulatory activity refers to the alteration of a drug’s effect when combined with another substance. Therefore, the combination of conventional antibiotics with a natural agent may enhance their biological activities due to compound interactions (Lima & Fernandes, 2022). When the combination enhances the antibiotic’s effect, it is termed a synergistic effect; if the activity is reduced or neutralized, it is considered antagonistic (De Souza et al.,2017).

Based on these considerations, this study aimed to evaluate the modulatory effect of *S. aromaticum* (clove) essential oil in combination with β-lactam antibiotics against Multidrug-Resistant *Staphylococcus aureus*.

MATERIALS AND METHODS

Isolation and Identification of Bacteria

Staphylococcus isolates available in the bacterial library of the Animal Health Laboratory at the Institute of Agricultural Sciences (ICA), Federal University of Minas Gerais (UFMG), obtained from mastitic milk of cows from farms in northern Minas Gerais, Brazil, were reactivated in BHI broth, purified, and confirmed their identifiy by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), following the methodology described by Assis et al. (2017). Identification was performed using the Microflex™ MALDI-TOF MS (Bruker Daltonics) at the Aquaculture Laboratory of the School of Veterinary Medicine, UFMG.

Susceptibility of *Staphylococcus aureus* to Beta-lactam Antibiotics

Strains identified as *S. aureus* were selected for multidrug resistance assessment using the disk diffusion method, following CLSI guidelines (2015a), on Mueller-Hinton agar. The beta-lactam antibiotics tested, obtained from Laborclin, were: Oxacillin (1 µg), Imipenem (10 µg), Meropenem (10 µg), Cefoxitin (30 µg), and Ampicillin + Sulbactam (10 µg).

The two antibiotics that showed the highest incidence of bacterial resistance in the disk diffusion test were selected for determination of MIC and MBC and modulatory analysis.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC and MBC of Oxacillin and Meropenem were determined to assess the lowest concentration required to inhibit or eliminate multidrug-resistant *S. aureus* strains. MIC was determined using the broth microdilution method in 96-well microtiter plates, based on CLSI (2015b) guidelines with modifications. Antibiotics were tested at concentrations of 1, 2, 4, 6, 8, 10, 12, 20, 30, 40, 50, 60, 70, 80, 90, 100, and 200 µL/mL. Bacterial suspensions were adjusted to a 0.5 McFarland standard (~10^8 CFU/mL), and 20 µL was added to each well. *S. aureus* ATCC 25923 was used as the positive control. All tests were conducted in triplicate. After incubation at 37°C for 24 hours, 1% 2,3,5-Triphenyltetrazolium chloride (TTC) reagent was added and incubated for an additional 2 hours.

Wells that showed no color change from yellow to red indicated bacterial inhibition. These strains were then plated on Plate Count Agar (PCA) to determine bactericidal activity. Plates were incubated at 37°C for 24 hours to assess bacterial viability (MBC).

Essential Oil Analysis by Gas Chromatography - Mass Spectrometry (GC-MS)

The essential oil of *S. aromaticum* (clove oil; OESA) was commercially obtained from Ferquima - Essential Oils Industry and Commerce, São Paulo, Brazil. Its chemical composition was analyzed at the Instrumental Chemistry Laboratory at ICA/UFMG, Montes Claros campus, following Farias et al. (2019).

Samples were subjected to chromatographic analysis. Compounds were identified by comparing mass spectra to the NIST 2.0 library (2009), using retention index (RI) values calculated according to Van Den Dool and Kratz (1963) and compared with literature data (Adams, 2012).

Determination of MIC and MBC for the OESA

The MIC of OESA was determined following the CLSI (2015b) guidelines with modifications. Oil concentrations of 320, 160, 80, 40, 20, and 10 µg/mL were tested in 96-well microplates. Bacterial suspensions were standardized to ~10^8 CFU/mL using the 0.5 McFarland scale, and added to the wells. After 24-hour incubation at 37°C, microbial growth was assessed based on color change of the TTC reagent.

The MIC was defined as the lowest concentration that inhibited visible bacterial growth. For MBC determination, aliquots from non-growth wells were plated on PCA, incubated at 37°C for 24 hours, and examined for bacterial proliferation. All tests were performed in triplicate.

Modulatory Effect of the Essential Oil Combined with Oxacillin and Meropenem

The modulatory effect was evaluated using the checkerboard assay, following the methodology described by Lahmar et al. (2016) with modifications. The assay involved multidrug-resistant *S. aureus* strains tested with binary combinations of Oxacillin and Meropenem. To enhance the simultaneous action

of the essential oil and antibiotics, one agent was serially diluted along the vertical axis and the other along the horizontal axis of the microplate (Figure I). A total of 49 different combinations of essential oil and antibiotics were tested. Each compound was tested starting from its MIC and diluted to 1/16, with bacterial inocula adjusted to 10^8 CFU/mL (0.5 McFarland). Plates were incubated at 37°C for 24 hours, followed by MIC reassessment. All assays were performed in triplicate.

The fractional inhibitory concentration index (FICI) was calculated as described by Siqueira et al. (2021), using the formula below:

$$FIC\ Essential\ oil = \frac{\text{MIC of combined oil}}{CIM\ do\ óleo\ essencial} + FIC\ \text{drug} = \frac{\text{MIC of combined antimicrobial}}{\text{MIC of antimicrobial alone}}.$$

FIGURE I

SOURCE: Siqueira et al., 2021

Data Analysis

The data obtained were subjected to descriptive analysis, including frequency, mean, and standard deviation calculations. Levene's test and the Shapiro-Wilk test were performed to assess the homogeneity and normality of the data, respectively. The mean inhibitory concentrations of clove essential oil and β-lactam antibiotics (used individually or in combination) were then compared using the non-parametric Kruskal-Wallis test. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using PAST software, version 4.17.

RESULTS

Out of 58 bacterial isolates, 24 were identified as *S. aureus* and used in this study. Antimicrobial susceptibility test revealed the highest frequency of resistance to the antibiotic combination Sulbactam + Ampicillin, observed in 23 out of 24 isolates (95.83%). This was followed by Meropenem in 21/24 (87.5%) isolates, Oxacillin in 19/24 (79.16%), Cefoxitin in 9/24 (37.5%), and Imipenem in 1/24 (4.16%), as shown in Figure II.

FIGURE II

All strains were resistant to two or more of the antibiotics tested (Table 1).

TABLE I

The essential oil used in the antibiotic modulation assays against multidrug-resistant *S. aureus* strains was properly characterized. Chromatographic analysis of the clove essential oil (CEO) identified Eugenol as the major compound (84.52%), followed by Caryophyllene and Eugenol acetate, as shown in Figure III.

FIGURE III

Figure 4 presents a heatmap of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for the essential oil and the antibiotics used in combination. The essential oil showed a MIC of 10 µl/ml against all *S. aureus* strains. The MBC was 20 µl/ml for 8 out of 24 samples and 40 µl/ml for 16 out of 24. For the antibiotics, greater variation was observed in both MIC and MBC values among the 24 isolates. The MIC of oxacillin ranged from 40 µl/ml (18/24) to 60 µl/ml (2/24). The MBC for oxacillin was determined for 15 out of 24 samples: 40 µl/ml in 6/15, 50 µl/ml in 2/15, 60 µl/ml in 4/15, and between 80–110 µl/ml in 1/15. The MIC of Meropenem ranged from 5 µl/ml (1/24) to 40 µl/ml (5/24). The MBC of Meropenem was determined for 19/24 isolates, ranging from 10 µl/ml (4/19) to 60 µl/ml (1/19).

FIGURE IV

When analyzing the MIC and MBC of the antimicrobials and clove essential oil against *S. aureus* strains, both MIC ($p = 0.0000$) and MBC ($p = 0.0004$) values for the essential oil were significantly lower than those of oxacillin, highlighting the oil's potency in combating Multidrug-resistant *S. aureus*. Meropenem also showed lower MIC ($p = 0.0000$) and MBC ($p = 0.0000$) values compared to oxacillin, but no significant difference was observed between Meropenem and the essential oil (Figure V).

FIGURE V

Figure 6 shows that the MICs of Oxacillin and Meropenem were significantly reduced when combined with clove essential oil (OXA - p = 0.0006; MER – p = 0.0012). Before combination testing, 10 µl/mL of OESA was required to inhibit bacterial growth. After the combination, this concentration decreased to 2.5 and 1.25 µl/mL. As for oxacillin, the required concentration dropped from 60 and 50 µl/mL to 3.75 and 3.12 µl/mL after the combination. Regarding Meropenem, the MIC was initially 40 and 20 µl/mL, which was reduced to 2.5 and 1.25 µl/mL when combined with the oil. These results suggest that clove essential oil positively modulates the inhibitory action of these antibiotics against *S. aureus* strains, enhancing their effect. Clove essential oil reduced the MIC of Oxacillin and Meropenem by 10-fold and 8-fold, respectively.

FIGURE VI

Among the 14 isolates selected for having the highest resistance (including the ATCC 25923 reference strain), the combination treatment showed an synergistic effect in all isolates, as shown in Table II. No antagonistic effects were observed for any strain, further supporting the modulatory potential of clove essential oil in combination with Oxacillin and Meropenem.

TABLE II

The contribution of clove essential oil in enhancing the antimicrobial effect against multidrug-resistant *S. aureus* strains is evident. The FIC of the essential oil differed from the FIC of the antibiotics (OXA - p = 0.0163; MER - p = 0.0091). Furthermore, the FICI of the antibiotics was significantly different from the FIC of each antibiotic alone (OXA - p = 0.0014; MER - p = 0.0003). As shown in Figure VII, the mean FICI of the combination of the essential oil with oxacillin and meropenem indicates a synergistic effect.

FIGURE VII

DISCUSSION

Multidrug-resistant *S. aureus* strains isolated from cows with subclinical mastitis pose a significant challenge in managing dairy herds, complicating treatment due to resistance to multiple antimicrobials. Phenotypic analyses

reveal that these bacteria frequently exhibit resistance to commonly used drugs such as Penicillin, Ampicillin, and Oxacillin, indicating β-lactam resistance. Additionally, resistance to other antimicrobials like Tetracycline, Erythromycin, and Gentamicin has also been reported, further complicating efforts to control infections caused by these strains (Algammal et al., 2020; El-Sayed & Kamel et al., 2021; Islam et al., 2023). A study conducted in Pakistan found that half of the *S. aureus* isolates from subclinical mastitis cases were resistant to multiple antibiotics (Haq et al., 2024). Research in Brazil supports this concern, with reports of phenotypic resistance to Penicillin and Tetracycline among subclinical mastitis isolates (Silva et al., 2023). These findings underscore the need for ongoing antimicrobial susceptibility monitoring and more effective strategies to control the spread of resistant strains on dairy farms.

Antimicrobial susceptibility testing (AST), also known as antibiotic sensitivity testing, is a crucial tool in identifying resistant and susceptible microorganisms, contributing to the global fight against bacterial resistance. In the present study, the highest resistance rates were observed for Ampicillin+Sulbactam, Meropenem, and Oxacillin, respectively. According to the study by Cominato et al. (2024), which evaluated market-available antibiotics from 2019 to 2024, β-lactams and aminoglycosides remain dominant. Meropenem and Imipenem, both carbapenems, belong to the broad-spectrum β-lactam class. These agents are not commonly used to treat bovine mastitis and should not be first-line treatments in humans either, except for infections involving multidrug-resistant organisms that respond specifically to these drugs. Meropenem shows superior in vitro activity against Gram-negative bacteria, while Imipenem is more active against Gram-positive organisms (Silva & Rodrigues Júnior, 2022), which may explain the greater sensitivity of *S. aureus* to Imipenem observed in some studies. Alarmingly, Meropenem resistance was detected in 87.5% of S. aureus isolates in this study - a rare finding for this species and suggestive of potential horizontal gene transfer from other resistant organisms, highlighting the urgent need for further investigation.

Cross-resistance among β-lactams was first reported in 1961 in S. aureus isolates from nosocomial infections (Jevons et al., 1961). The first methicillin- or oxacillin-resistant *S. aureus* (MRSA/ORSA) strain isolated from animals was reported in 1972 in milk from mastitic cows (Devriese et al., 1972).

In a study conducted by Carvalho et al. (2021) in Brazil's Federal District, 45.45% of *S. aureus* isolates from mastitic milk were resistant to Ampicillin+Sulbactam. Antimicrobial resistance is a global concern. For example, Savita et al. (2017) in India reported 100% resistance to Ampicillin+Sulbactam among 53 evaluated strains, while Tallat et al. (2023) reported the highest resistance to Oxacillin (96.7%), followed by Ampicillin and Ampicillin+Sulbactam (66.7%) across three Egyptian provinces. β-lactams function by binding to penicillin-binding proteins (PBPs), disrupting bacterial cell wall synthesis. The mecA gene, located in the SCCmec element, encodes a modified PBP (PBP2a/PBP2'), which has low affinity for β-lactams and allows Staphylococcus spp. to continue cell wall synthesis even in the presence of inhibitory antibiotic concentrations (Livermore, 2000; Paterson et al., 2014a).

Regarding OESA, this study identified eugenol as the major component. Eugenol, a phenolic compound, has been widely studied and shown to be effective against *S. aureus* due to its strong bactericidal activity, which involves disrupting bacterial cytoplasmic membranes and reducing the likelihood of resistance development (Resende et al., 2017; Gomes et al., 2018). Essential oils are complex mixtures of volatile compounds extracted from plants, and their chemical composition can vary significantly across species and even plant parts. These oils typically contain terpenes, phenols, and other organic compounds that confer their characteristic aroma and biological activity. This chemical complexity contributes to their antimicrobial properties, making essential oils a promising alternative to synthetic antibiotics. Research has demonstrated that essential oils are effective against various bacterial strains, including resistant ones, providing a more natural treatment option and helping to reduce reliance on conventional antibiotics (Swamy et al., 2016; Visan & Negut et al., 2024).

Borborema et al. (2022) used Gas Chromatography - Mass Spectrometry (GC-MS), the same analytical method used in this study, to quantify volatile oil components and found 79.4% eugenol. Santos et al. (2017) also reported similar findings - 84% eugenol, 6% caryophyllene, and 8% eugenyl acetate - using steam distillation of flower buds from the same supplier as the current study. Bantim et al. (2020), using hydrodistillation, found comparable results: 83.9% eugenol, 11.53% eugenyl acetate, and 3.57% caryophyllene. These

differences in composition may be attributed to the distinct extraction methods used. As noted by Busato et al. (2014), the chemical profile of essential oils can vary considerably depending on the extraction technique employed.

Eugenol exhibits notable antimicrobial activity, particularly against *S. aureus*. Studies have shown that eugenol effectively inhibits Multidrug-resistant *S. aureus* growth by disrupting bacterial membranes and interfering with essential enzymatic functions (Das et al.,2016). Xu et al. (2016) reported that clove oil (OESA), containing 76.23% eugenol, showed strong antibacterial activity against *S. aureus* ATCC 25923, with a minimum inhibitory concentration (MIC) of 0.625 mg/mL. Additionally, eugenol has been shown to inhibit biofilm formation and reduce bacterial adhesion, further reinforcing its potential as an antimicrobial agent. Its effectiveness against *S. aureus* highlights eugenol as a promising candidate for developing new therapeutic strategies to combat antibiotic-resistant infections (Marchese et al., 2017).

MIC and MBC testing in this study revealed that even at low concentrations (20-40 µg/mL), OESA exhibited bactericidal activity. Several other studies using different extraction methods have confirmed the antimicrobial effects of OESA against *S. aureus*. Guimarães et al. (2017), using OESA from a different supplier, reported an MIC of 60 µg/mL - six times higher than the current study - likely due to the lower eugenol (65%) and eugenyl acetate (3–5%) content. Pereira (2017), using hydrodistillation, reported an MIC of 25 µg/mL, similar to our findings. Supporting the current results, Borborema et al. (2022), using oil from the same supplier and extraction method, reported an MIC of 10 µg/mL and MBC of 20 µg/mL against *S. aureus*.

The combination of essential oils with conventional antibiotics has gained attention for enhancing antimicrobial efficacy. OESA demonstrated significant synergistic effects when combined with traditional antibiotics. Reports show that OESA and other eugenol-rich oils, when combined with antibiotics such as Ampicillin or Gentamicin, enhanced antibacterial activity against oral pathogens, reducing antibiotic MICs (Moon et al., 2011). Similarly, studies have shown that both OESA and pure eugenol possess significant inhibitory effects against various foodborne microorganisms, suggesting their potential as natural antimicrobial agents (Hu et al., 2018). Moreover, the research by Marchese et al. (2017) discusses the antimicrobial action of eugenol and eugenol-containing

essential oils, offering mechanistic insights into their effects. These findings support the potential of OESA and eugenol to boost the efficacy of conventional antibiotics, offering promising avenues to combat resistant microbial strains.

The evaluation of the interaction between the essential oil and conventional antimicrobials aimed to enhance their antibacterial properties. The Checkerboard method has proven to be the most effective approach for assessing antibacterial potential (Figure I). However, it is important to note that this research was conducted using in vitro assay techniques, and the results require validation through in vivo experimental models to confirm findings with greater accuracy (Santos et al.,2020).

Combination therapy is one of the most effective tactics for dealing with antimicrobial resistance, seeking synergistic interaction against a wide variety of bacteria. Combination therapy has proven crucial in antimicrobial treatment for several reasons: It prevents drug resistance; it enhances activity through the use of components with additive or synergistic activity; it reduces effective doses, lowering the cost/toxic effect; and it broadens the spectrum of activity. However, the combination of two or more agents can result in synergistic, additive, partial, and antagonistic effects (Sharma et al., 2023). In our study, 100% synergism was obtained in multidrug-resistant strains of *S. aureus*, a promising result for human and veterinary pharmaceuticals, aiming at new therapeutic approaches to bacterial diseases.

Synergistic effects were also found in the research by Mbaveng & Kuete (2017), in which *S. aromaticum* extract demonstrated synergism with several antibiotics, including tetracycline, chloramphenicol, erythromycin, vancomycin, penicillin, oxacillin, cephalothin, cefixite, cotrimoxazole, and ofloxacin, in *S. aureus*.

The observed synergy may result from the inhibition of a common biochemical pathway or from increased cell permeability, leading to enhanced antimicrobial activity. Several studies support this concept. Notably, Yang et al. (2017) investigated the combined effect of cinnamon bark essential oil and Meropenem, observing that the additive effect of the essential oil enhanced antibiotic uptake by the bacteria. It is believed that components of the essential oil -particularly eugenol, found in OESA - may disrupt bacterial cell membranes by interacting with phospholipids, opening membrane channels and facilitating

antibiotic entry. This membrane disruption increases the permeability of the bacterial cell wall, allowing antimicrobial agents to reach their intracellular targets more efficiently (Swamy et al., 2016; Batiha et al., 2020; Elbestawy et al., 2023).

Eugenol's ability to act as a membrane permeabilizer has been highlighted in several studies, including research on its role in biofilm inhibition and its synergy with conventional antibiotics. Its action on cell membranes is attributed to its ability to alter lipid bilayers, causing leakage of cellular contents and bacterial cell death. Furthermore, studies suggest that the antimicrobial activity of eugenol can be enhanced when combined with other agents, resulting in a synergistic effect that not only increases antibiotic efficacy but may also reduce the required dosage for effective treatment, thereby minimizing potential side effects (Ashrafudoulla et al., 2020; Ulanowska & Olas, 2021).

These findings reinforce the potential of OESA - particularly due to its eugenol contente - as a promising natural adjuvant to conventional antibiotics, boosting their effectiveness against bacterial infections through membrane disruption and enhanced permeability (Bai et al., 2022; Kowalewska & Majewska-Smolarek, 2023).

CONCLUSION

OESA was able to modulate the action of antibiotics, exerting a synergistic effect in inhibiting multidrug-resistant pathogenic strains. These findings indicate that it is possible to reduce antibiotic dosages when combined with clove essential oil to achieve effective antibacterial activity against multidrug-resistant *S. aureus* strains. The results are promising for the use of plant-derived compounds as alternative options to help minimize the problem of microbial resistance.

Further studies are needed to investigate the interaction of essential oils with other antimicrobials and against other bacterial species, as well as to assess potential toxicity, with the goal of supporting future efforts to combat bacterial resistance. Molecular analyses are recommended to better elucidate the interaction mechanisms between clove essential oil and beta-lactam antibiotics.

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AUTHORS' CONTRIBUTIONS

Ellem Cristina Gomes Damascena, Cintya Neves De Souza, Anna Christina de Almeida: Study design and conception. Ellem Cristina Gomes Damascena, Leonardo Ferreira Oliveira, Agueda Maria de França Tavares, Adriana Fróes Do Nascimento Souto, Eliane Macedo Sobrinho Santos: Drafted the manuscript, data analysis and laboratory analysis. Leonardo Ferreira Oliveira, Agueda Maria de França Tavares, Hércules Otacílio Santos, Eliane Macedo Sobrinho Santos: Literature review, statistical analysis, and manuscript formatting. Ellem Cristina Gomes Damascena, Adriana Fróes Do Nascimento Souto: Collected samples and data. All authors have read, reviewed, and approved the final manuscript.

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FIGURE CAPTIONS

Figure I - Dilution of antibiotics and essential oils used in the Checkerboard method

Figure II – Frequency of *S. aureus* strains resistant to the antimicrobials ASB (Sulbactam + Ampicillin), OXA (Oxacillin), MER (Meropenem), CFO (Cefoxitin), and IPM (Imipenem).

Figure III - Chemical composition of the essential oil from *Syzygium aromaticum* (clove).

Figure IV - Heatmap showing the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of clove essential oil (CEO) and the antibiotics Oxacillin and Meropenem.

Figure V - Average minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of antimicrobials against different *S. aureus* strains.

Figure VII - Comparison of the average MIC of antibiotics alone and in combination with clove essential oil.

Figure VII – Comparison between FICI and FIC values of clove essential oil and the antibiotics Oxacillin and Meropenem. Average FICI of the combination of clove essential oil with Oxacillin and Meropenem showing an additive effect.

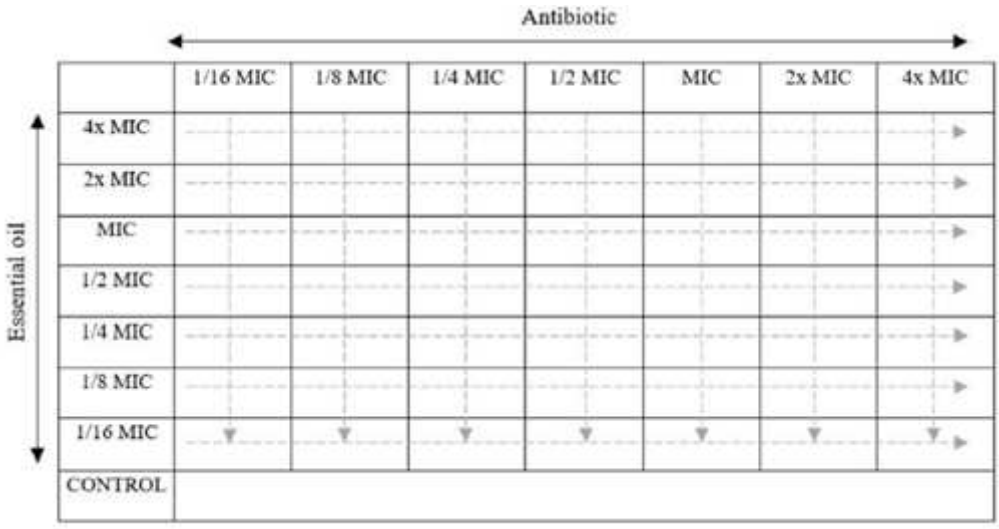

Figure I

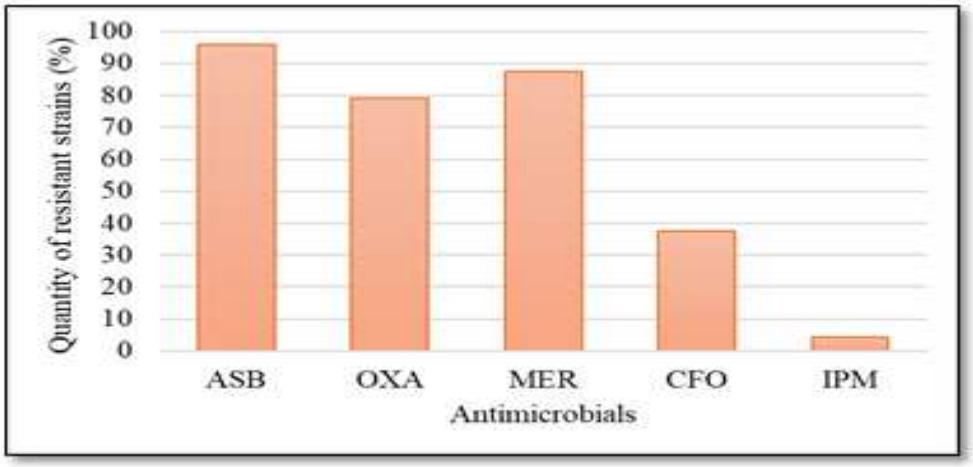

Figure II

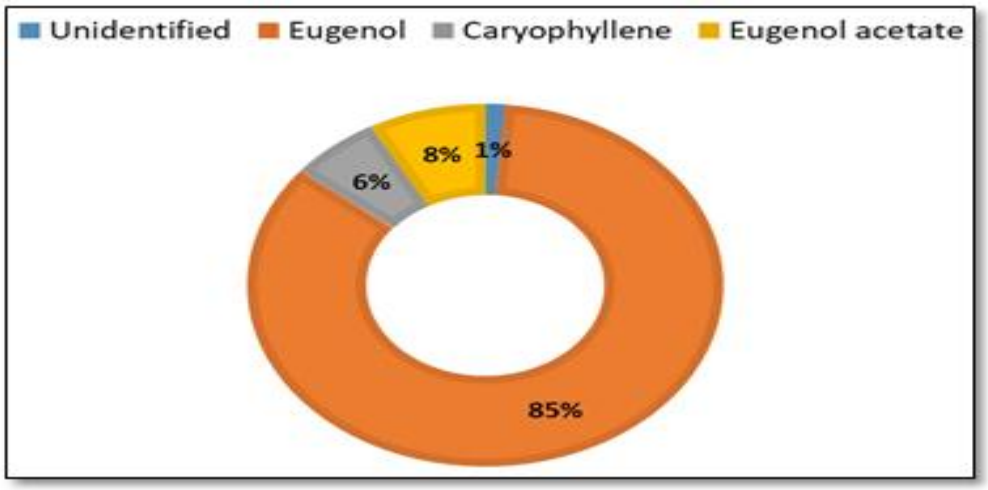

Figure III

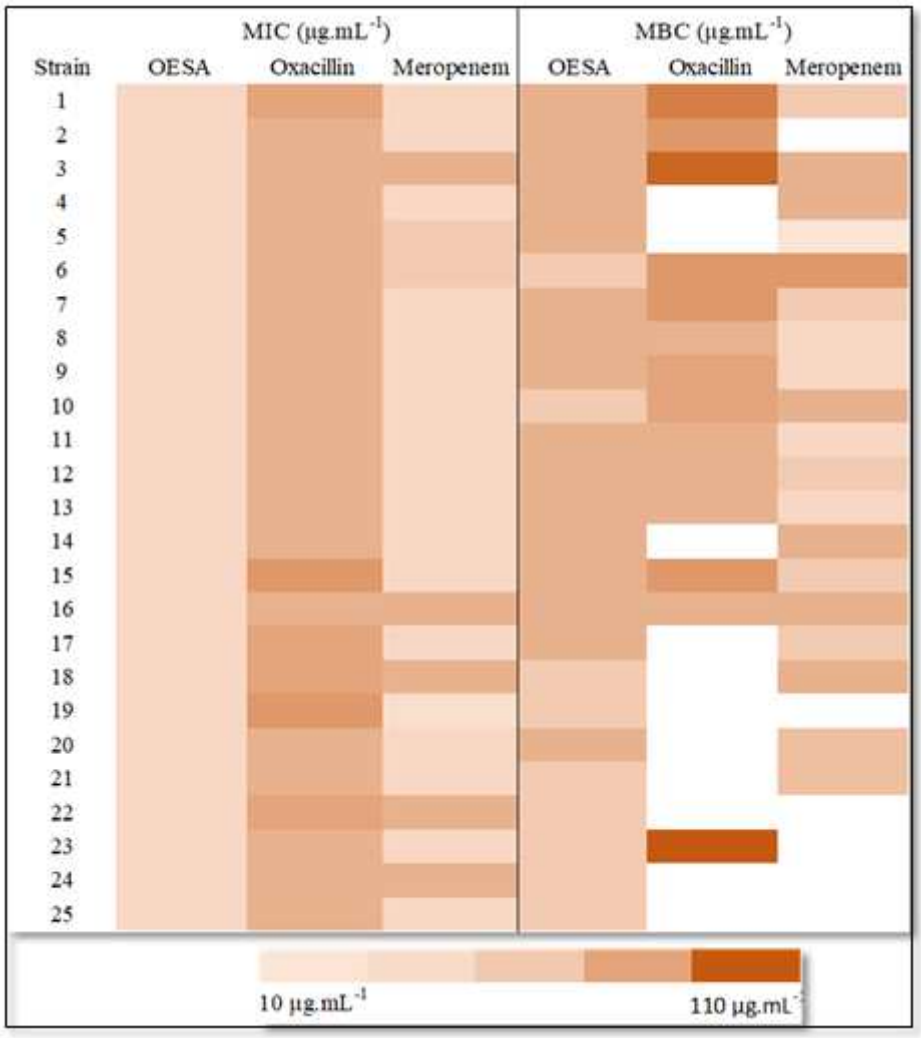

Figure IV

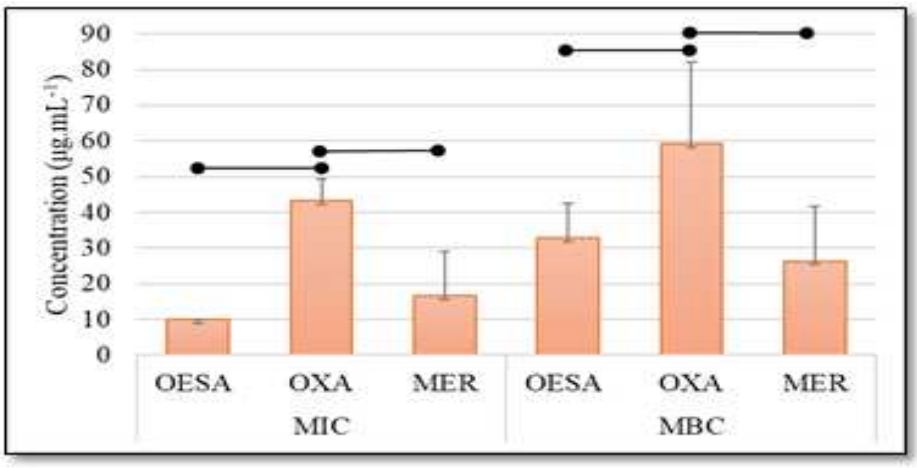

Figure V

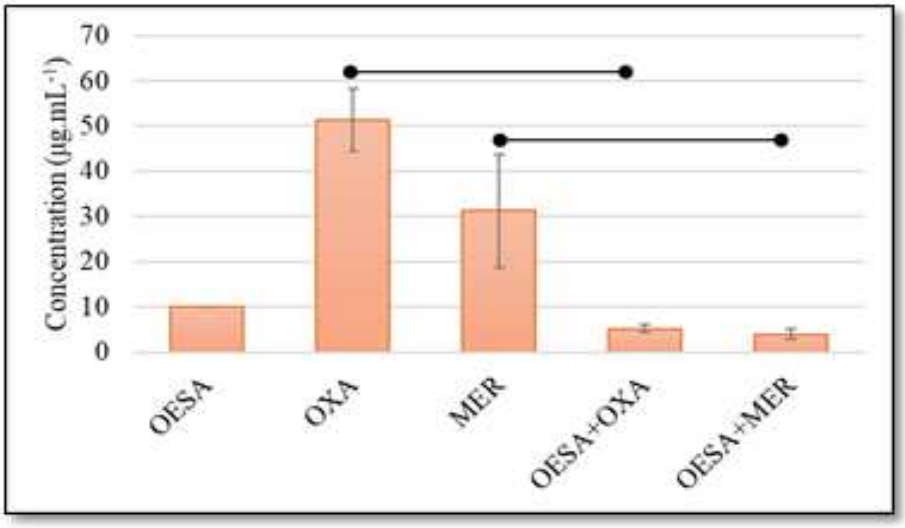

Figure VI

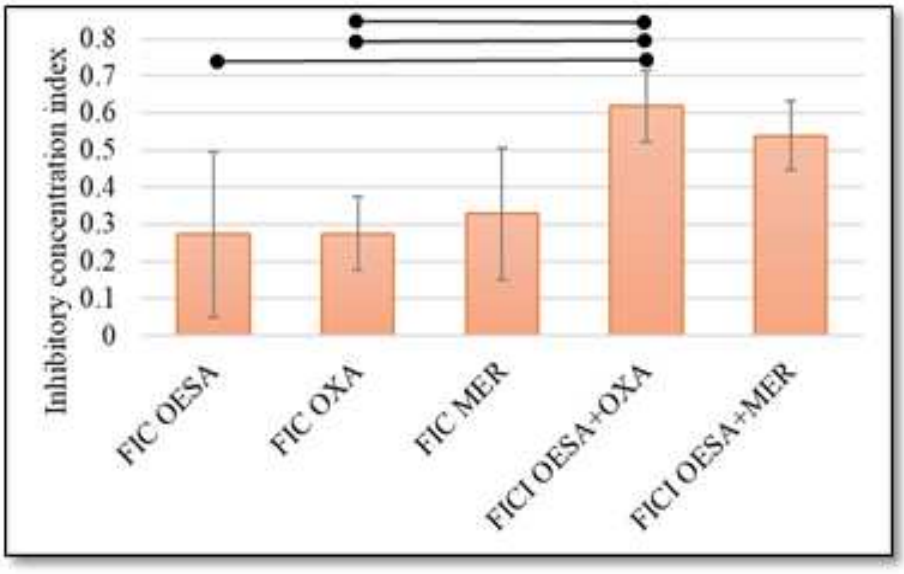

Figure VII

6. ARTIGO CIENTÍFICO 2- EFFECT OF *SYZYGIUM AROMATICUM* MICROENCAPSULATED ESSENTIAL OIL COMBINED WITH BETA-LACTAM ANTIMICROBIALS ON MULTIDRUG-RESISTANT *STAPHYLOCOCCUS AUREUS* STRAINS

Formato de acordo com as normas da revista: International Journal of One Health

ABSTRACT

Background and Aim: Microencapsulation is an emerging and promising trend to enhance the antimicrobial action of essential oils, helping to minimize the resistance of microorganisms to conventional antibiotics. The aim of this study was to evaluate the effect of the microencapsulation of *Syzygium aromaticum* essential oil (OESA) combined with beta-lactam antimicrobials on the inhibition of multi-resistant strains *of Staphylococcus aureus*. In addition, we sought to understand the role of the whey protein isolate WPI 90%, used in the wall of the microcapsules, in potentiating the antimicrobials, by means of bioinformatic analysis.

Materials and Methods: Whey protein isolate WPI 90% and gum arabic were used as coating materials in the OESA microencapsulation process using the complex coacervation technique. The inhibitory activity of microencapsulated OESA (OESAM), associated or not with beta-lactam antibiotics, was tested against 10 isolates of multidrug-resistant strains of *S. aureus*. Bioinformatic analysis was carried out using the STRING online platform to investigate the mechanisms of action of microcapsules in potentiating the antimicrobial action of OESA.

Results: A significant difference was observed in the minimum inhibitory concentrations of the antibiotics oxacillin and meropenem alone and combined with OESAM ($p<0.05$). There was an average reduction of 12 and 51 $\mu g.ml^{-1}$ in the minimum inhibitory concentration of OESAM when combined with the antibiotics oxacillin and meropenem, respectively. There was an average

reduction in antimicrobial doses of up to 2.7 times when combined with OESAM. Although in some strains of *S. aureus an* indifferent effect was observed between OESAM and the antibiotics, the effects of synergism and additivity stood out. Bioinformatics analysis revealed 3 clusters in the protein interaction network. Cluster 1 is represented by the tryptophan biosynthesis pathway. Cluster 2 is characterized by the siderophore biosynthesis pathway. Cluster 3 is defined by the phosphorelay signal transduction system.

Conclusions: This study has demonstrated the potential of using microencapsulated OESA combined with beta-lactam antibiotics to combat multidrug-resistant strains of *S. aureus*, mitigating the resistance mechanisms of these bacteria to conventional antimicrobials.

Keywords: Bacterial resistance. Natural antimicrobial. Synergism. WPI 90%. Molecular mechanism.

INTRODUCTION

Antibiotic resistance is increasing all the time. This is one of the main factors responsible for the increase in morbidity and mortality and hospital admissions, and is considered a global threat to public health [1]. Antimicrobial resistance impacts humans, animals and ecosystems. In this scenario, the One Health approach is key to developing sustainable solutions. The World Health Organization (WHO) and the Ministry of Health highlight action plans aimed at monitoring and controlling resistance, especially in the agricultural sector [2].

In dairy herds, resistant strains of *S. aureus* have been responsible for hindering the treatment and control of bovine mastitis. This disease occurs predominantly in the subclinical form, although acute cases, such as gangrenous mastitis with lethal potential, can also occur [3]. *S. aureus* stands out for causing persistent infections, with a high tendency to chronicity and a low cure rate, even after antibiotic therapy [3]. *S. aureus* strains have shown a remarkable ability to develop resistance to practically all the antimicrobials used in clinical therapy, especially beta-lactam antibiotics, which are widely used to treat infections caused by Gram-positive bacteria [4]. These antibiotics act by

inhibiting essential enzymes involved in the biosynthesis of peptidoglycan, which is a fundamental structural component of the bacterial cell wall, such as transpeptidases, transglycosylases and carboxypeptidases [5].

Recognized by the WHO as one of the greatest contemporary health challenges, antimicrobial resistance significantly outstrips the speed at which new drugs are developed. In recent decades, public interest in natural therapies has increased and the use of herbal medicines is growing. In this sense, essential oils (EOs) have been widely investigated as a promising alternative to synthetic medicines, mainly due to their natural origin, lower environmental impact and distinctive therapeutic properties [6]. The essential oil of *Syzygium aromaticum* (cloves) has been widely used in various sectors, such as traditional medicine and the food industry, due to the presence of bioactive compounds with multiple therapeutic properties [7]. Among the main constituents of clove essential oil are eugenol, β-caryophyllene, α-humulene and eugenol acetate, which have antioxidant, antibacterial, anti-inflammatory, antihypertensive and potential anticancer activities [8].

Essential oils are liquid and volatile at room temperature [6]. In this context, microencapsulation has been highlighted as a strategic technology to achieve greater chemical, oxidative and thermal stability [9]. In addition, this technique contributes to extending shelf life, maintaining biological and functional activity, improving physicochemical properties and controlling the release of active compounds [10]. The microencapsulation process is based on the separation of the coating material from a liquid medium, resulting in the coating of the core material dispersed in the medium [11]. Various materials such as proteins and polysaccharides can be used as coating materials in the production of microcapsules. Based on the available scientific evidence, the microencapsulation of essential oils is an emerging and promising trend for various industrial applications [6]

In view of this, the aim of this study was to evaluate the effect of the microencapsulation of *Syzygium aromaticum* essential oil combined with beta-lactam antimicrobials on the inhibition of multidrug-resistant *S. aureus* strains. In addition, we sought to understand the role of the whey protein isolate WPI 90%, used in the wall of the microcapsules, in potentiating the antimicrobials, by means of bioinformatic analysis.

MATERIALS AND METHODS

Obtaining strains of multidrug-resistant *S. aureus*

We used isolates of the genus *Staphylococcus* from the bacterioteca of the Animal Health Laboratory of the Institute of Agrarian Sciences (ICA) of the Federal University of Minas Gerais (UFMG), Montes Claros campus - MG. Ten isolates of the *S. aureus* species were selected. To identify the strains, the previously frozen isolates were reactivated in BHI (Brain Heart Infusion) broth and then plated on TSA (Tryptic Soy Agar) agar. Pure colonies with the characteristic morphology of Gram-positive cocci grouped in clusters were selected. The fresh and pure cultures were subjected to identification by MALDI-TOF mass spectrometry, using the Microflex™ MALDI-TOF MS equipment (Bruker Daltonics), at the Aquaculture Laboratory of the Veterinary School of the Federal University of Minas Gerais (UFMG), in Minas Gerais, Brazil [12].

In order to confirm Beta-lactam resistance, the strains identified by MALDI TOF as *S. aureus* were evaluated using the disc diffusion technique on Mueller-Hinton agar using the following antibiotics: oxacillin (1mcg), imepenem (10mcg), meropenem (10mcg), cefoxitin (30 mcg) and sulbactam+ ampicillin (10 mcg) [13].

Obtaining and characterizing the essential oil of *Syzygium aromaticum* (OESA)

The OESA used in this study was previously purchased from Ferquima Indústria e Comércio Ltda® and characterized by chromatography. The chemical composition was analyzed at the Instrumental Chemistry Laboratory at ICA/UFMG - Montes Claros campus, using gas chromatography coupled to mass spectrometry (GC-MS), using a model 7890 chromatographic system. The compounds were identified by comparing the mass spectra with data from the NIST 2.0 library (2009), and based on the relative retention indices (RI), which were determined experimentally and compared with values reported in the literature [14,15].

Microencapsulation of OESA using the complex coacervation technique

Whey protein isolate WPI 90% and gum arabic were used as coating materials in the OESA microencapsulation process using the complex coacervation technique. The technique was optimized in terms of pH, where a phmeter was used for this evaluation, and citric acid to obtain a suitable pH. The ideal pH and proportion of whey for complex coacervation is 3.75 and 3:1, respectively [16].

The OESA was microencapsulated and then freeze-dried to produce solid microcapsules. The oxidative stability, yield and microencapsulation efficiency of the surface oil and morphology of these solid microcapsules were determined, following the methodology with modifications described by Eratte et al [16].

Yield of the microencapsulation process

The freeze-dried microcapsules were weighed on an analytical balance to determine the yield of the process, using equation (I):

Equation (I)

Yield of microcapsules % : $\frac{Mass\ of\ lyophilized\ microcapsules\ (g)}{Total\ mass\ of\ whey + gum\ arabic + oil(g)}\ \ x\ \ 100$

Microencapsulation efficiency

Encapsulation efficiency was determined by quantifying the surface essential oil and the total content present in the microcapsules. To do this, 3 g sample of microcapsules was added to 30 mL of hexane and subjected to orbital shaking for 5 minutes at 225 rpm. The mixture was then filtered through filter paper with a pore size of 5 µm and the solid residue was washed three times with 10 mL of hexane to completely extract the surface oil. The resulting filtrate was concentrated in a rotary evaporator at 60 °C to remove the solvent and recover the oil. The sample was then kept under hood exhaust for 4 hours to eliminate the residual hexane.

The total content of encapsulated essential oil was determined using the acid digestion method described by Eratte et al. (2014), with adaptations. To do this, 3 g of microcapsules were added to 30 mL of 4N HCl solution and stirred at

225 rpm for 15 minutes. Afterwards, 15 mL of hexane was added to the mixture, which was stirred again for 18 hours at room temperature, allowing the oil to be extracted completely. The resulting solution was centrifuged at 24,471 × g at 10 °C for 30 minutes using a Sorvall ST 16R centrifuge (Thermo Scientific, Waltham, USA). The organic phase (hexane), containing the dissolved oil, was recovered and concentrated in a rotary evaporator at 60 °C. Finally, the sample was kept under hood exhaust for 4 hours to eliminate the residual hexane.

The percentages of surface oil (SO), total oil (TO) and microencapsulation efficiency (ME) were determined using equations (II), (III) and (IV) respectively.

$$SO=\frac{Ws}{Wm} \quad x \quad 100\% \qquad \text{(Equation II)}$$

$$TO=\frac{Wt}{Wm} \quad x \quad 100\% \qquad \text{(Equation III)}$$

$$ME=\frac{Wt-Ws}{Wt} \quad x \quad 100\% \qquad \text{(Equation IV)}$$

Where Wt and Ws are the mass values (g) of the total and surface oil of the microcapsules and Wm is the mass (g) of the microcapsules.

Determination of the Minimum Inhibitory Concentration (MIC) of OESAM

The Minimum Inhibitory Concentration (MIC) of OESAM was determined according to the methodology described by the Clinical and Laboratory Standards Institute, 2015b, with adaptations [13]. Solutions at concentrations of 170 µg, 150 µg, 130 µg, 110 µg and 90 µg of OESAM were prepared and applied to microtiter plates. The bacterial strains were standardized at 10^8 CFU/mL, using the McFarland 0.5 scale, and applied to the wells of the microplates, which were incubated at 37 °C for 24 hours. The MIC was read using the change in color of 1% 2,3,5-triphenyl tetrazolium chloride (TTC), which shows the presence of bacterial growth.

Determination of the MIC of the Antibiotics Oxacillin and Meropenem

The antibiotics oxacillin and meropenem were evaluated for their MIC against multidrug-resistant strains *of S. aureus*. The MIC was determined using

the broth microdilution method, using 96-well microtiter plates, according to the CLSI protocol (2015b), with adaptations [13]. Increasing concentrations of the antimicrobials were tested (1, 2, 4, 6, 8, 10, 12, 20, 30, 40, 50, 60, 70, 80, 90, 100 and 200 µL/mL). The bacterial strains were standardized based on the Mc Farland 0.5 scale (approximately 10^8 CFU/mL) and 20 µL were inoculated into each well. The *S. aureus* ATCC 25923 strain was used as a positive control. All tests were carried out in triplicate. After incubation at 37 °C for 24 hours, 1% TTC was added and then incubated for 2 hours. The absence of a change in the color of the TTC (from yellow to red) indicated inhibition of bacterial growth.

Checkerboard microdilution method of OESAM associations with the antibiotics Oxacillin and Meropenem

Modulatory activity was assessed using the *Checkerboard* method as described by Lahmar *et al* [17], with modifications. The analysis was conducted between multidrug-resistant *S. aureus* strains with double combinations of Oxacillin and Meropenem. In order to enhance the joint action between the essential oil and the antibiotic, the first antimicrobial agent in the combination was serially diluted along the ordinate (vertical axis) of the microplate, while the second agent was serially diluted along the abscissa (horizontal axis). A total of 49 different combinations of antibiotic concentrations were tested in association with the essential oil. The concentrations were established from the MIC of each compound, with successive dilutions up to 1/16, using a bacterial inoculum standardized at 10^8 UFC/mL using the Mc Farland 0.5 scale. The plates were incubated at 37 °C for 24 hours, after which the new MIC for each combination was determined. All tests were carried out in triplicate.

The Fractional Inhibitory Concentration Index (FICI) was used to interpret the results, according to the methodology proposed by Siqueira et al. (2021) [18]. The FICI was calculated by adding up the minimum fractional inhibitory concentrations (FICs) of the compounds tested (Synergy: FICI ≤0.5; Additivity: FICI >0.5 to 1; Indifference: 1 to 4; Antagonism: FICI>4).

The experiments were conducted in triplicate using two independent analyses and the results were expressed as means and standard deviation.

Bioinformatic analysis

In order to evaluate the possibility of potentiating the antimicrobial effect of the essential oil through microencapsulation, a protein interaction network was developed to analyze the action of the microcapsules on *S. aureus*. The microcapsules are made up of the active material , which is the essential oil of *Syzygium aromaticum*, and the wall material made up of gum arabic (GA) and whey protein isolate (WPI 90%), registered with the MAP under No. 0102/1328 - NCM 3502.20.00 - SAP P100103 and P700103. The amino acids present in the composition of WPI 90% (Aspartic Acid, Glutamic Acid, Alanine, Arginine, Cysteine, Phenylalanine, Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Proline, Serine, Tyrosine, Threonine, Tryptophan, Valine) were entered into the STRING V. 12.0 platform [19], selecting the *S. aureus* species. The previously formed protein interaction network, with a confidence score of 0.4 and all interaction sources selected, was expanded to contain no more than 10 interactors in the first layer and a maximum of 30 interactors in the second layer. The network was divided into 3 groups using K-means clustering. Functional enrichment of the network was obtained via KEGG.

The network was analyzed based on the scientific literature and the results obtained *in the in vitro* tests.

Statistical analysis

The data obtained in the in vitro tests had a normal distribution and were statistically analyzed using the ANOVA method supplemented by the Tukey test with a significance level of 5% ($p \leq 0.05$).

RESULTS

Obtaining efficient microspheres requires attention to basic criteria such as understanding the general properties of microcapsules. As for the coating material, the morphological and dimensional characteristics of the microcapsules, carried out by optical microscopy (Zeiss microscope coupled to the image capture system), can be seen in figure 1. Variations can be seen in the morphology, size, regularity, appearance and dispersion of the microcapsules, which in some cases showed the formation of lumps. The stability of the microcapsules in terms of pH was obtained in the 3.75 range.

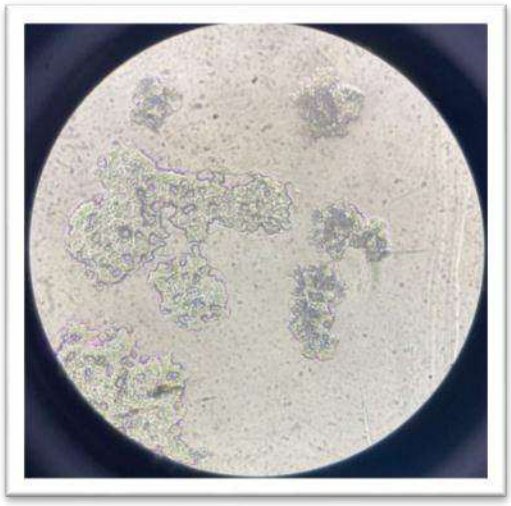

Figure 1- Formation of microcapsules at pH 3.75 under a 40x objective.

The main bioactive component present in the oil used was eugenol (85%). With regard to the performance of the OESAM microencapsulation process, a yield of 39.11% was obtained.

Figures 2 and 3 show the inhibitory potential of OESAM in combination with the antibiotics oxacillin and meropenem, respectively. Figure 2 shows a significant difference in the minimum inhibitory concentrations of the antibiotic oxacillin alone and in combination with OESAM ($p<0.05$). These results are encouraging in terms of reducing the dose of the antibiotic to achieve the desired effect, which could contribute to a reduction in the resistance of antimicrobials conventionally used in the treatment and control of bovine mastitis.

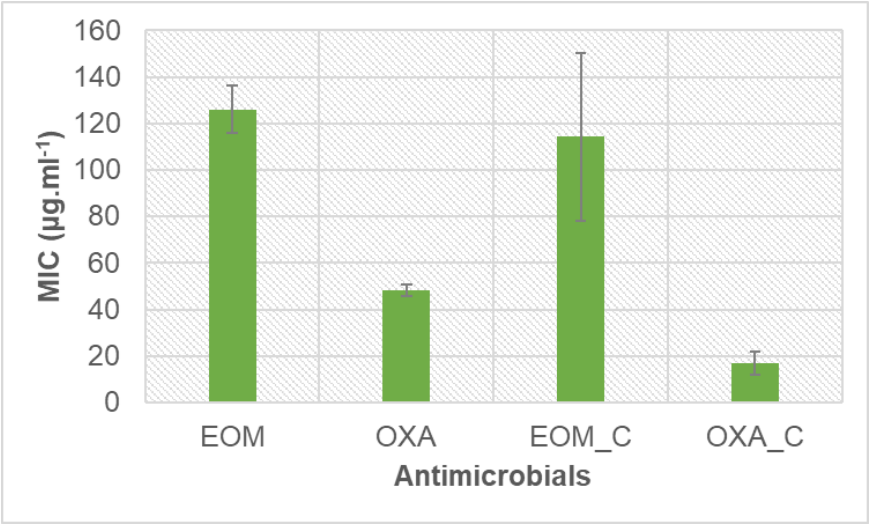

Figure 2 - Minimum inhibitory concentration of OESAM and oxacillin, alone and in combination

Even more prominently, the minimum inhibitory concentration of the antibiotic meropenem was significantly reduced in the presence of OESAM ($p<0.05$), as shown in figure 3.

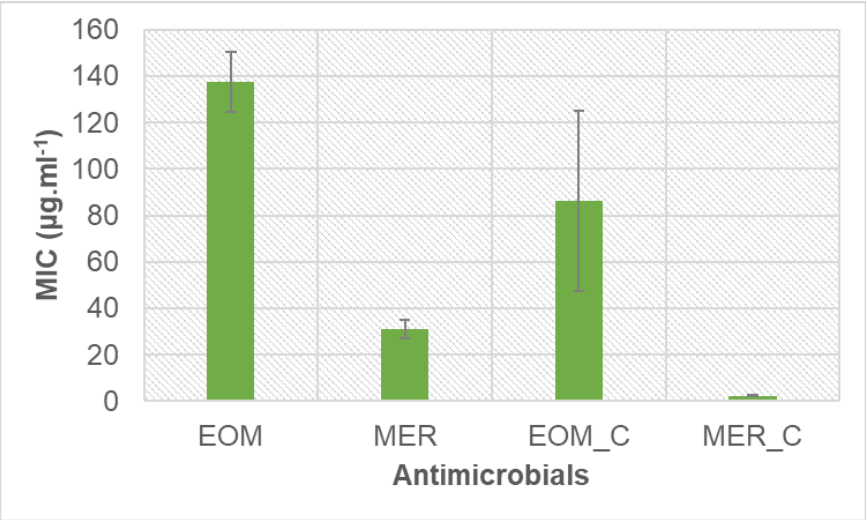

Figure 3 - Minimum inhibitory concentration of OESAM and meropenem, alone and in combination.

The results are promising, since there was an average reduction of 12 and 51µg.ml^{-1}in the minimum inhibitory concentration of OESAM when combined with the antibiotics oxacillin and meropenem, respectively. Also surprisingly, the average reduction in the minimum inhibitory concentration of oxacillin and meropenem when combined with OESAM was 31 and 29µg.ml-1, respectively (Figure 4A). In this sense, there was an average reduction in antimicrobial doses of up to 2.7 times (Figure 4B) when combined with OESAM.

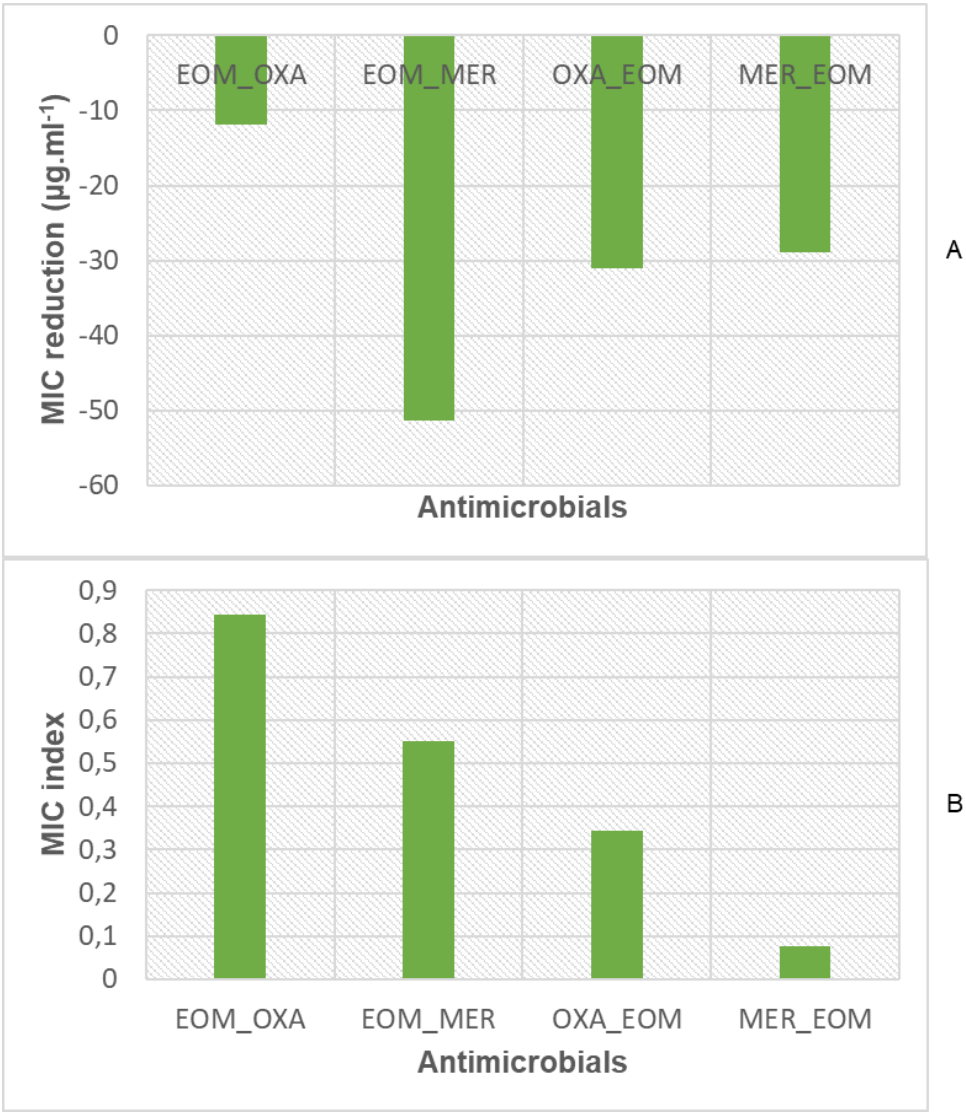

Figure 4 - Effect of combining microencapsulated clove essential oil with antimicrobials on the minimum inhibitory concentration. A - Reduction in the minimum inhibitory concentration due to the combination of clove essential oil and antimicrobials. B - Index obtained by the minimum inhibitory concentration of the combination divided by the minimum inhibitory concentration of the isolate.

The fractional inhibitory concentration indices that determine the types of effects between OESAM and the antibiotics are shown in figures 5A and 6A for oxacillin and meropenem, respectively. Although in some strains of *S. aureus an* indifferent effect was observed between OESAM and antibiotics, the effects of synergism and additivity stood out (Figures 5B and 6B), indicating an important joint action between natural and chemical antimicrobials.

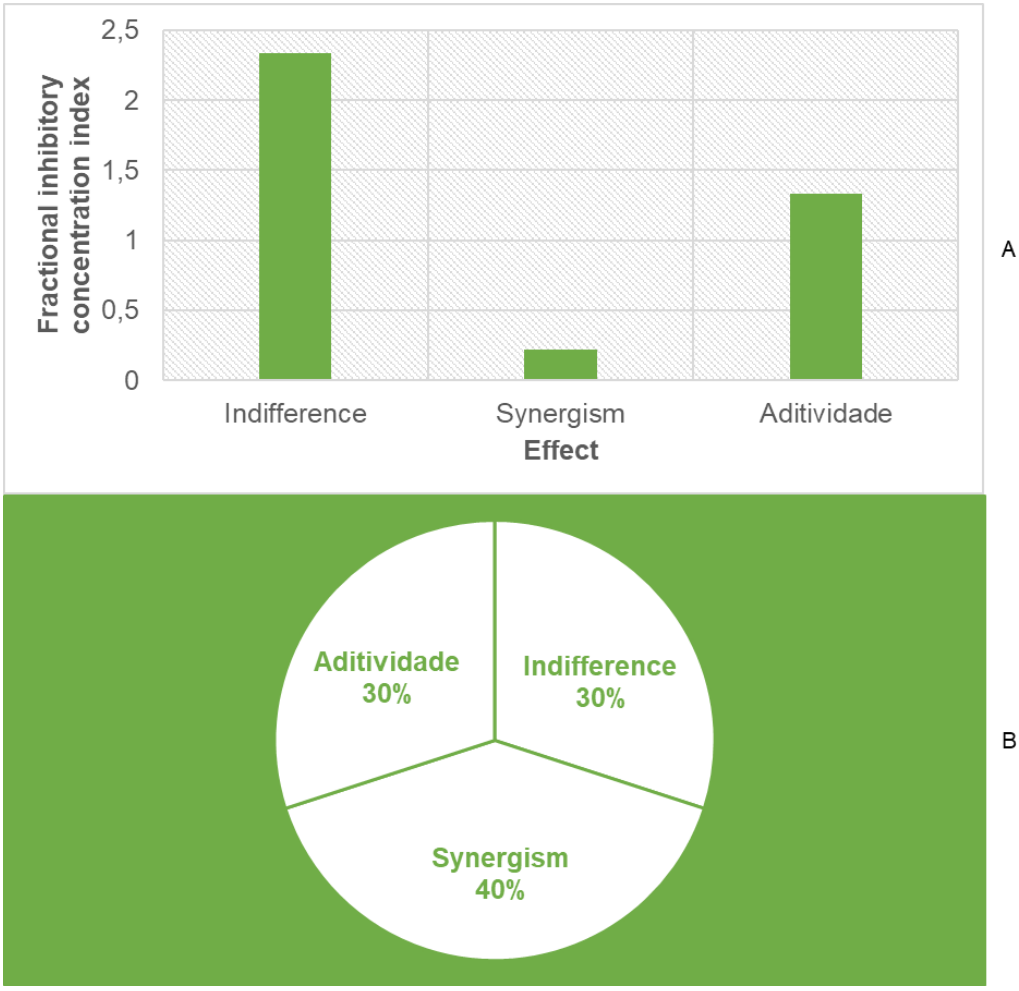

Figure 5 - Types of effects caused by the combination of microencapsulated clove essential oil and oxacillin. A - Index of the fractional inhibitory concentration obtained for each type of effect. B - Quantitative number of multidrug-resistant *Staphylococcus aureus* strains showing each type of effect.

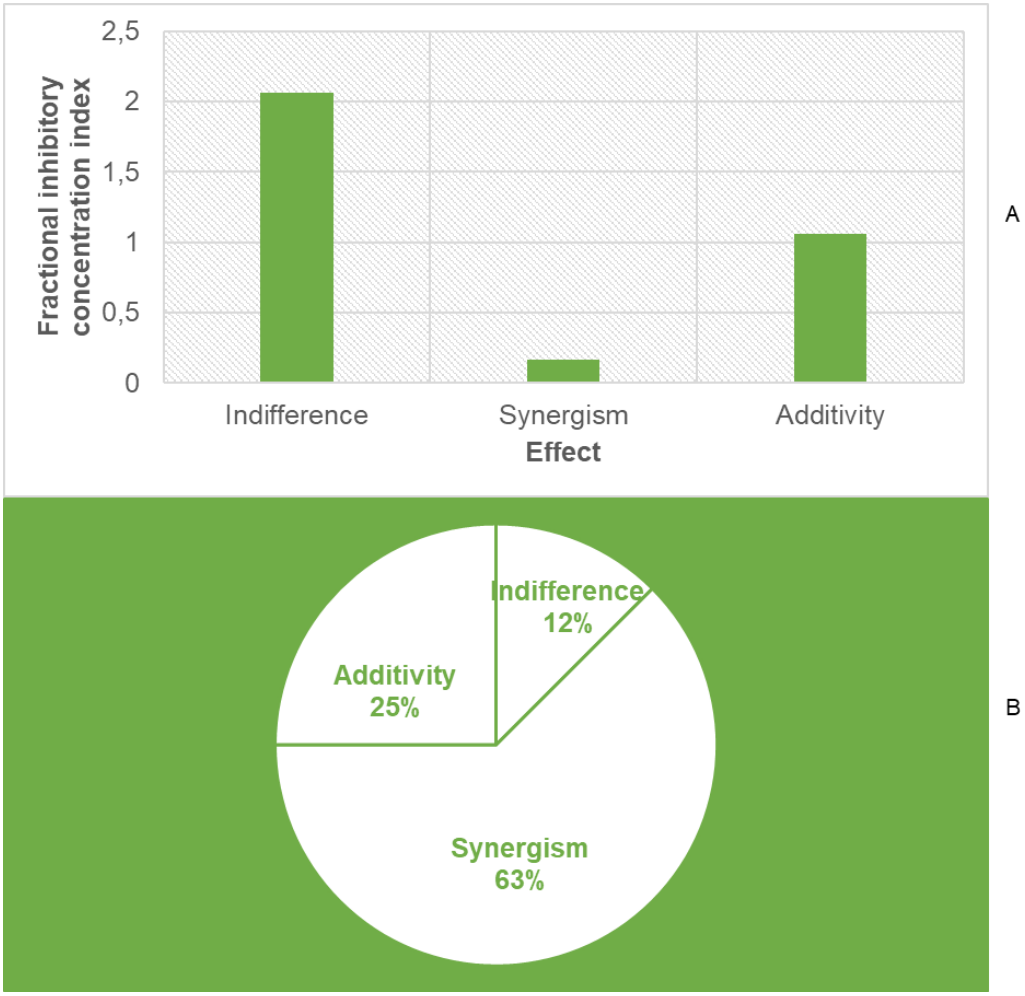

Figure 6 - Types of effects caused by the combination of microencapsulated clove essential oil and meropenem. A - Index of the fractional inhibitory concentration obtained for each type of effect. B - Number of multidrug-resistant *Staphylococcus aureus* strains that showed each type of effect.

Figure 7 shows the protein interaction network formed from the amino acids present in the 90% WPI in the *S. aureus* species. It can be seen that the network is organized into 3 distinct clusters (represented by red, blue and green). This is important because it reveals molecular, biological and functional mechanisms in these clusters.

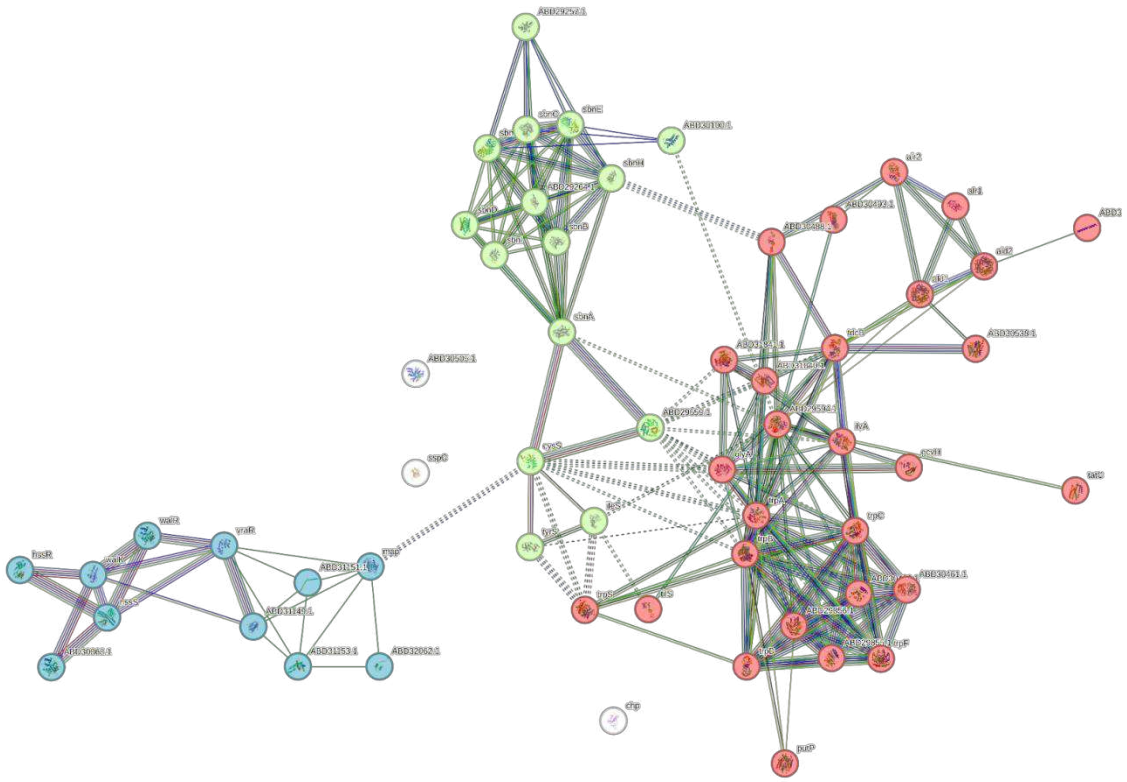

Figure 7 - Protein interaction network obtained on the STRING V.12.0 platform from the amino acids present in the whey protein isolate (WPI) used as the basis for formulating microcapsules for the *Staphylococcus aureus* species.

The mechanisms involved in each of the clusters of the protein interaction network are shown in Table 1. Cluster 1 (red) is represented by the tryptophan biosynthesis pathway. Cluster 2 (green) is characterized by the siderophore biosynthesis pathway. Cluster 3 is defined by the phosphorelay signal transduction system.

Table 1 - Division of network proteins into groups, with the respective description of each group.

Color	Cluster Id	Gene count	Description
	Cluster 1	28	Tryptophan biosynthesis
	Cluster 2	15	+ Siderophore biosynthesis
	Cluster 3	11	+ Phosphorelay signal transduction system

Source: STRING V.12.0.

In the functional enrichment analysis of the protein interaction network, using the KEGG pathway, it can be seen that most of the proteins in the network act in the amino acid biosynthesis, secondary metabolite biosynthesis and metabolic pathways (Figure 8).

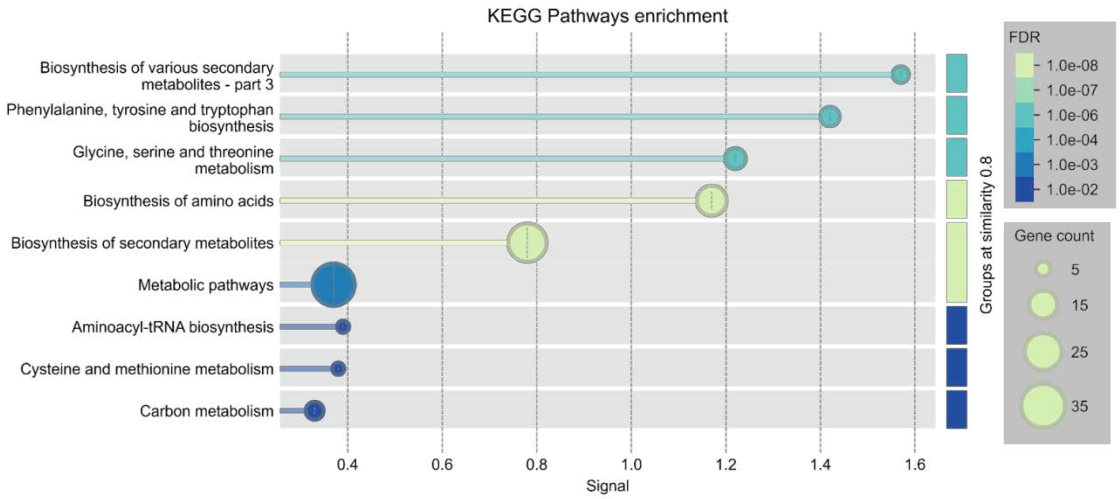

Figure 8 - Functional enrichment of the protein interaction network extracted from the STRING V.12.0 platform.

DISCUSSION

The analyses carried out to characterize the microcapsules containing OESA were carried out in order to assess the effectiveness and stability of the microencapsulation. The maintenance of the structural integrity of the microcapsules observed after the process indicates that they were adequately rigid, a crucial factor for protecting the OESA against external agents such as oxygen, light radiation and thermal variations. This characteristic contributes significantly to the physicochemical stability of the encapsulated compound [21]. Regarding the yield of microcapsules, in the present study we obtained 39.11% yield. This value is comparable to that reported by Carvalho *et al*. who, using the same complex coacervation methodology and the same active compound, obtained a yield of 45.03%. This similarity suggests that the technique employed has satisfactory reproducibility [20] .

Efficiency is one of the most important quality parameters for microencapsulated oils [22]. Some hypotheses can be suggested to explain the

low efficiency of 40% in the present study. The technological parameters of the freeze-drying stage can significantly influence the efficiency of the process, requiring careful optimization. Xiao *et al*. [23] investigated the complex coacervation of lemon essential oil (LEO) using different formulation conditions and technological variables, employing gum arabic and type B gelatine as encapsulating agents. The authors reported encapsulation efficiency ranging from approximately 14% to 66%, as determined by UV-VIS spectrophotometry. They suggested that part of the losses observed could be associated with volatilization during the emulsification and coacervation stages [23]. Many studies have shown that emulsions with smaller droplet sizes result in microcapsules with greater microencapsulation efficiency by increasing the viscosity which favors the rapid formation of the crust around the particle during drying [24].

The addition of gelatine, in addition to gum arabic (GA) and sodium caseinate, promoted a significant increase in the retention of volatile compounds, as reflected by the increase in encapsulation efficiency (EE) from 28.6% to 65.9% in the study by Zhang *et al* [25]. These results imply that the technological parameters and composition of the encapsulating matrices have a critical influence on the efficiency of the microencapsulation process by complex coacervation, especially when associated with freeze-drying, and reinforce the importance of choosing the right biopolymers to stabilize emulsions and reduce volatilization losses. These findings suggest that optimizing technological and formulation variables not only improves process efficiency, but also the stability and functional performance of microcapsules, which is essential for applications in food, pharmaceutical or veterinary systems.

Considering the loss of bioactive compounds present in essential oils, the microencapsulation process has emerged as an effective strategy to protect them against volatilization, oxidative degradation and environmental variations, as well as allowing the controlled and targeted release of active ingredients, preserving their functional activity [26]. In the research carried out by Koc *et al*. [27], both the free oil and the microencapsulated clove essential oil demonstrated the ability to inhibit the growth of Gram-positive bacteria, with the exception of *Bacillus cereus*, at the same concentration [27]. In relation to the results found by Cruz-Valenzuela et al. the microencapsulated essential oil

demonstrated antibacterial activity against *Escherichia coli*, *Salmonella enterica* subsp. *enterica* serovar *Choleraesuis*, *Listeria monocytogenes* and *S. aureus*, showing a minimum inhibitory concentration (MIC) of 5.5 mg/mL for all the strains tested. To assess the impact of the oil on bacterial growth parameters, a sub-inhibitory concentration corresponding to half the MIC (2.75 mg/mL) was used [28].

Further studies indicate that, in addition to the intrinsic activity of essential oils (EOs), the influence of surfactants - such as Tween 20, Tween 80 and Triton X-100 - and solvents, such as ethanol, present in the test media should be considered. These emulsifiers play an important role in facilitating the penetration of antimicrobial compounds into the structures of the bacterial cell wall and membrane. In this way, both surfactants and solvents can interfere with the efficacy of EOs or the response of microorganisms, influencing the results obtained in sensitivity tests [29]. In the case of Gram-positive bacteria, such as *S. aureus*, there is a thicker layer of peptide glycans, giving the cell greater rigidity and protection. However, it is speculated that this layer may facilitate interaction with the coating layers of the microcapsules. In this way, the *S. aureus* bacteria could be more efficiently eliminated by releasing the oil directly closer to its wall, which facilitates the interaction/approach of the gum arabic and WPI 90% particles. This would result in an increase in the local concentration of the oil, and therefore greater efficiency of the antimicrobial function [30].

The results obtained from the drug interaction tests, involving 10 bacterial isolates including the standard strain *Staphylococcus aureus* ATCC 25923, revealed that the association between the antimicrobials tested resulted in a synergistic effect in 50% of the cases. This data is relevant, as it suggests that the combination of agents, possibly including essential oils and antibiotics, can potentiate antimicrobial activity against resistant strains, representing a promising strategy for combating bacterial multidrug resistance. In addition , 27.7% of the isolates showed an additive effect, i.e. the combination of compounds resulted in an increase in antimicrobial activity, although to a lesser extent than synergism. This finding reinforces the importance of investigating antimicrobial associations as an alternative therapy. On the other hand, 22.3% of the isolates showed an indifferent response to the combination of

compounds, which indicates that the action of one compound does not interfere with the action of the other. Taken together, these data show that assessing the interaction profile between bioactive compounds and antibiotics is essential for targeting more effective therapeutic strategies, especially in the context of infections caused by resistant bacteria. The use of tests such as the *checkerboard*, combined with the calculation of the fractional inhibitory concentration index (FICI), is essential for selecting combinations with clinical potential.

Bioinformatic analysis, as shown in the protein interaction network in figure 6, is important, since protein interaction networks are useful in understanding the molecular mechanisms involved in specific conditions [31]. The three clusters observed in the protein interaction network reveal distinct mechanisms of action of WPI 90% on multidrug-resistant strains of S. aureus. The clusters function as a set of proteins with a greater degree of interconnection in relation to the whole network, and may represent specific molecular, biological and functional mechanisms [32].

With regard to cluster 1, represented by the tryptophan biosynthesis pathway, it is well established in the literature that tryptophan catabolites, such as indole, play an important role in microorganisms, affecting spore formation, plasmid stability, drug resistance, biofilm formation and virulence [33]. Studies have reported the antimicrobial activity of this metabolite against *Staphylococcus aureus* and other bacteria [34,35]. In addition, the literature review carried out by Roager and Licht. showed that microbial tryptophan catabolites resulting from proteolysis can influence the health of the host, suggesting that these metabolites activate the immune system [36].

Cluster 2, characterized by the siderophore biosynthesis pathway, suggests that *S. aureus* can use this pathway as a resistance mechanism by acquiring nutrients such as iron [37]. The bacteria synthesize siderophores, such as staphylopherrin A and staphylopherrin B, which bind strongly to iron and are recognized by specific membrane receptors, which enable the bacteria to assimilate the iron-bound complexes [38]. Therefore, by blocking this mechanism, microencapsulation can potentiate the effect of clove essential oil, which has a strong antioxidant action due to the majority presence of eugenol,

which acts as a ferric ion chelator, resulting in the prevention of hydroxyl radical formation [39].

The phosphorelay signal transduction system, defined in cluster 3, allows us to understand bacterial signal transduction through the phosphorylation of regulators [40]. In order for bacteria such as *S. aureus* to carry out their biological functions, the phosphorylation of their response regulators is often necessary. In this sense, any factor that interferes with the phosphorelay signal transduction system can prevent the bacteria from acting [41,42].

The amino acid biosynthesis pathways, secondary metabolite biosynthesis and metabolic pathways obtained by analyzing the KEGG pathway of action of WPI 90% reveal that the results obtained *in the in silico* analyses support the hypothesis that the amino acid constituents of WPI 90% can potentiate OESAM action by interfering with these pathways of action of *S.aureus* the study therefore shows that the results obtained in the in silico potentiate OESAM action by interfering with these pathyways of action of S.*aureus*. The study therefore shows that the results obtained in the in silico.

The recommended next steps include in vivo investigations, pharmacokinetic evaluations, toxicity studies and the development of specific formulations for clinical application and the search for new techniques to improve myencapsulation efficiency.

CONCLUSION

This study has demonstrated the potential of using OESAM in combination with beta-lactam antibiotics to combat multidrug-resistant strains of *S. aureus*. In general, the microencapsulation of clove essential oil enhances its antimicrobial effect, promoting greater stability, controlled release of the active compounds and increased efficacy against multidrug-resistant bacterial strains, which reinforces its potential as a therapeutic adjuvant in veterinary medicine. More studies are needed in an attempt to improve the efficiency of oil microencapsulation.

Recommended next steps include *in vivo* investigations, pharmacokinetic evaluations, toxicity studies and the development of specific formulations to help reduce antimicrobial resistance.

COMPETING INTERESTS

The authors declare that they have no competing interests

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7. CONSIDERAÇÕES FINAIS

- A eficiência de 40% obtida na microencapsulação do OESA evidencia a viabilidade da técnica, embora ressalte a necessidade de otimização das condições experimentais para maximizar o aproveitamento do composto ativo;
- A análise cromatográfica do OESA revelou teor de 85% de eugenol, confirmando sua elevada pureza e potencial biológico, o que reforça sua relevância como composto ativo de interesse farmacológico;
- O Óleo essencial de Cravo-da-índia puro foi capaz de potencializar a ação de antibióticos betalactâmicos exercendo efeito sinérgico em 100% das cepas de *Staphylococcus aureus* multirresistentes testadas;
- A microencapsulação do óleo essencial de cravo promoveu efeito sinérgico, potencializando o efeito dos antibióticos Oxacilina e Meropeném frente as cepas multirresistentes de *S.aureus*;
-Foram observados efeitos aditivos, sugerindo a possibilidade de redução das concentrações dos antimicrobianos quando associados ao óleo encapsulado;
-Houve casos de indiferença em um número restrito de isolados testados, o que sugere a aplicabilidade de estudos posteriores sobre outras espécies microbianas;
- As análises in sílico evidenciaram que as vias de biossíntese de aminoácidos, a biossíntese de metabólitos secundários identificadas pela análise KEGG do WPI 90%, corroboram a hipótese de que seus constituintes pontecializam a ação do OESAM no combate a bactérias multirresistentes.

8. ANEXO I – COMPROVANTES DE SUBMISSÕES DOS ARTIGOS 1 E 2

Submission Confirmation

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Thank you for your submission

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Date Submitted
18-Jun-2025

Dear Ellem Cristina Gomes Damascena,

You are co-author in an article submitted to **International Journal of One Health** and entitled **Effect of microencapsulation of Syzygium aromaticum essential oil combined with beta-lactam antimicrobials on multidrug-resistant Staphylococcus aureus strains** (Manuscript Number: IJOH-2025-07-038).

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If you think that you should not be one of the authors in this manuscript, please contact the editorial office (editor@onehealthjournal.org).

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